conjugating the first monoclonal antibody to the second monoclonal antibody, wherein the first and second monoclonal antibodies have anti-neoplastic activity in a conjugated form and have substantially no anti-neoplastic activity in an unconjugated form.

43. (Three times amended) A pharmaceutical composition comprising a homoconjugate comprising a monoclonal antibody and a pharmaceutically acceptable carrier, wherein the monoclonal antibody does not comprise an Fc region and wherein the monoclonal antibody is an anti-CD19, anti-CD20, anti-CD21, anti-CD22, anti-breast tumor, anti-ovarian tumor, anti-prostate tumor, anti-lung tumor, or anti-αHer2 monoclonal antibody and wherein the monoclonal antibody has anti-neoplastic activity in a conjugated form and has substantially no anti-neoplastic activity in an unconjugated form.

REMARKS

Entry of the amendments is believed proper given that they will substantially reduce the number of issues on appeal and place certain claims in condition for allowance.

The Advisory Action withdrew rejections of claims 4, 5, 16, 17, 46, and 47 under 35 U.S.C. § 102(b) as anticipated by Ahlem *et al.*, U.S. Patent 5,273,743. See Advisory Action page 2, paragraph 1. Despite the assertion of the Advisory Action that "No claims are allowed," (Advisory Action page 5, line 11), and the assertion on the cover sheet that these claims are rejected for purposes of appeal, no grounds of rejection are of record against these claims.

After entry of both Amendments, the subject matter of former claims 4, 5, 16, 17, 46 and 47 is in independent claim format with all other claims dependent to them. There are no other rejection of claims covering this subject matter. Therefore, because all rejections have been withdrawn with respect to the subject matter of these claims, Applicants respectfully submit that the current claims stand allowable upon entry of both amendments. Because all of the remaining

rejected claims depend upon these amended claims, their rejections should be moot upon entry of the amendment. Therefore, entry of this Second Amendment will reduce the number of issues on appeal to simply whether or not the claims after entry of the Second Amendment are allowable.

The examiner is invited to contact the undersigned at the telephone number listed below with any questions.

Respectfully submitted,

Mark B. Wilson
Reg. No. 37, 259
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 536-3035

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United States Patent [19]

Bagshawe et al.

[11] Patent Number: 5,683,694

[45] Date of Patent: Nov. 4, 1997

[54] **METHOD FOR THE TREATMENT OF TUMORS WITH CONJUGATED ANTIBODY A5B7 AND A PRODRUG**

[75] Inventors: **Kenneth D. Bagshawe**; **Gordon T. Rogers**; **Surinder K. Sharma**, all of London, United Kingdom

[73] Assignee: **Cancer Research Campaign Tech. Ltd. & Zeneca Ltd.**, London, England

[21] Appl. No.: **465,624**

[22] Filed: **Jun. 5, 1995**

Related U.S. Application Data

[62] Division of Ser. No. 313,866, Sep. 28, 1994, which is a division of Ser. No. 642,301, Dec. 21, 1990, abandoned.

[30] **Foreign Application Priority Data**

Apr. 22, 1988 [GB] United Kingdom 8809616

[51] **Int. Cl.**[6] **A61K 39/42**; C07K 16/28; C07K 19/00

[52] **U.S. Cl.** **424/178.1**; 424/181.1; 424/138.1; 435/183; 530/387.7; 530/388.8; 530/391.7

[58] **Field of Search** 424/178.1, 181.1, 424/138.1; 435/183; 530/387.7, 388.8, 391.7

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,185,111	1/1980	Ducep et al. .
4,564,675	1/1986	Kurabayashi et al. .
4,675,187	6/1987	Konishi et al. .
4,762,707	8/1988	Jansen et al. .

FOREIGN PATENT DOCUMENTS

1216791	1/1987	Canada .
089880	9/1983	European Pat. Off. .
0142905	5/1985	European Pat. Off. .
140728	5/1985	European Pat. Off. .
186551	7/1986	European Pat. Off. .
287353	10/1988	European Pat. Off. .
WO87/03205	6/1987	WIPO .
WO88/07378	10/1988	WIPO .

OTHER PUBLICATIONS

L Hellström et al., "Monoclonal Mouse Antibodies Raised against Human Lung Carcinoma", *Cancer Research*, 46, pp. 3917–3923 (Aug. 1986).

G. W. Philpott et al., "Affinity Cytotoxicity of Tumor Cells with Antibody–Glucose Oxidase Conjugates, Peroxidase, and Arsphenamine", *Cancer Research*, 34, pp. 2159–2164 (Sept 1974).

L Hellström et al., "Monoclonal Antibodies to Two Determinants of Melanoma–Antigen p97 Act Synergistically in Complement–Dependent Cytotoxicity", *The Journal of Immunology*, 127(1), pp. 157–160 (Jul. 1981).

V. J. Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery", *Directed Drug Delivery*, pp. 247–267 (1985).

T. Nishiyama et al., "Antineoplastic Effects in Rats of 5–Fluorocytosine in Combination with Cytosine Deaminase Capsules", *Cancer Research*, 45, pp. 1753–1761 (Apr. 1985).

D. E. V. Wilman, "Prodrugs in Cancer Chemotherapy", *Biochemical Society Transactions*, 14, pp. 375–382, (615th Meeting, Belfast 1986).

P. J. O'Dwyer et al., *The New England Journal of Medicine*, 312, pp. 692–700 (Mar. 14th, 1985).

A. P. Albino et al., "Heterogeneity in Surface Antigen and Glycoprotein Expression of Cell Lines Derived from Different Melanoma Metastases of the Same Patient", J. Exp. Med., 154, pp. 1764–1778 (Dec. 1981).

R. Arnon et al., "In Vitro and In Vivo Efficacy of Conjugates of Daunomycin with Anti–Tumor Antibodies", *Immunological Rev.*, 62, pp. 5–27, (1982).

K. D. Bagshawe, "Antibody Directed Enzymes Revive Anti–Cancer Prodrugs Concept", *Br. J. Cancer*, 56, pp. 531–532 (Nov. 1987).

K. D. Bagshawe, "A Novel Approach to Prodrug Activation Using a Monoclonal Antibody Conjugated to Carboxypeptidase G2", from the Third International Conference on Monoclonal Antibody Immunoconjugates for Cancer, Abstract #43, p. 70 (San Diego, Feb. 4–6, 1988).

R. W. Baldwin et al., "Design and Therapeutic Evaluation of Monoclonal Antibody 791T/36–Methotrexate Conjugates" in *Monoclonal Antibodies and Cancer Therapy*, pp. 215–231 (Alan R. Liss, Inc. 1985).

R. W. Baldwin et al., "Monoclonal Antibodies in Cancer Treatment", *Lancet*, pp. 603–605 (Mar. 15, 1986).

R. W. Baldwin et al. "Monoclonal Antibody Drug Conjugagates for Cancer Therapy", *Monoclonal Antibodies in Cancer: Advances in Diagnosis and Treatment*, Jack A. Roth (ed)., pp. 215–257 (Futura Publishing Co., 1986).

J. P. Brown et al., "Structural Characterization of Human Melanoma–Associated Antigen p97 with Monoclonal Antibodies", *J. Immunology*, 127 (2), pp. 539–546, (Aug. 1981).

E. A. Clark et al., "Role of the Bp35 Cell Surface Polypeptide in Human B–Cell Activation", *Proc. Natl. Acad. Sci.*, 82, pp. 1766–1770 (Mar. 1985).

S. T. Crooke et al. (eds.) *Anthracyclines: Current Status and New Developments*, Academic Press, cover pages and table of contents, (New York, 1980).

(List continued on next page.)

Primary Examiner—Lila Feisee
Attorney, Agent, or Firm—Spencer & Frank

[57] **ABSTRACT**

A method for treatment of tumors to which antibody A5B7 binds is disclosed. A5B7 is a monoclonal antibody which binds to carcinoembryonic antigen. The method comprises administering to a host in need of such treatment an effective amount of a system which comprises a conjugate comprising: antibody A5B7 or a humanized version of A5B7 linked to an enzyme selected from the group consisting of carboxypeptidase G2 (CPG2), nitroreductase, a human enzyme, a non-human enzyme rendered non-immunogenic in man and a humanized catalytic antibody; and a prodrug convertible under the influence of the conjugate into a cytotoxic drug.

24 Claims, 2 Drawing Sheets

OTHER PUBLICATIONS

R. A. DeWeger et al., "Eradication of Murine Lymphoma and Melanoma Cells by Chlorambucil–Antibody Complexes", *Immunological Rev.*, 62, pp. 29–45 (1982).

M. J. Embleton et al., "Antibody Targeting of Anti–Cancer Agents", *Monoclonal Antibodies for Cancer Detection and Therapy*, R. W. Baldwin and V.S. Byers (eds.), pp.321–322 (Academic Press, 1985).

M. J. Embleton, "Targeting of Anti–Cancer Therapeutic Agents by Monoclonal Antibodies", *Biochemical Society Transactions*, 14, pp. 393–395 (615th Meeting, Belfast 1986).

N. Endo et al. "In Vitro Cytotoxicity of a Human Serum Albumin–Medicated Conjugate of Methotrexate with Anti–MM46 Monclonal Antibody", *Cancer Research*, 47, pp. 1076–1080 (Feb. 15th, 1987).

P. J. Fraker et al., "Protein and Cell Membrane Iodinations with a Sparingly Soluble Chloroamide, 1,3,4, 6–Tetrachloro–3a,6a–Diphenylglycoluril", *Biochem. Bio–physical Research Comm.*, 80(4), pp. 849–857 (Feb. 1978).

I. Hellström et al., "Antitumor Effects of L6, an IgG2a Antibody that Reacts with Most Human Carcinomas", *Pro.Natl.Acad.Sci.U.S.A*, 83, pp. 7059–7063 (Sep. 1986).

I. Hellström et al., "Antibodies for Drug Delivery", *Controlled Drug Delivery* (2nd ed.), Robinson and Lee (eds.), pp. 638–642 (1987).

P. L. Ipata et al., "Baker's Yeast Cytosine Deaminase. Some Enzymic Properties and Allosteric Inhibition by Nucleosides and Nucleotides", *Biochemistry*, 10(23) pp. 4270–4276 (1971).

T. Katsuragi et al., "Affinity Chromatography of Cytosine Deaminase from *Escherichia coli* with Immobilized Pyrimidine Compounds", *Agric. Biol. Chem.*, 50(7), pp. 1713–1719 (1986).

J. M. Lambert et al., "Purified Immunotoxins That Are Reactive with Human Lymphoid Cells", *J. Biol. Chemistry* 260 (22), pp. 12035–12041 (1985).

J. P. Mach et al., "Improvement of Colon Carcinoma Imaging: From Polyclonal Anti–CEA Antibodies and Static Photoscanning to Monoclonal Fab Fragments and ECT", *Monoclonal Antibodies for Cancer Detection and Therapy*, R. W. Baldwin et al (eds), pp. 53–64, Academic Press (1985).

R. B. McComb et al. (eds)., *Alkaline Phosphatase*, Plenum Press, Chapters 4, 5, 6 and 11 (New York 1979).

R. G. Melton et al., "In Vivo Localization of Carboxypeptidase G2:Antibody Conjugates in Human Colon Carinoma Xenografts", from the Third International Conference on Monoclonal Antibody Immunoconjugates for Cancer Abstract #83, p. 110 (San Diego, Feb. 4–6, 1988).

S. Monfardini et al. (eds), *Manual of Cancer Chemotherapy*, Third Edition, UICC Technical Reports Series, 56, Table of Contents, pp. 1–230 (Geneva 1981).

F. L. Moolten et al., "Antibodies Conjugated to Potent Cytotoxins as Specific Antitumor Agents", *Immunological* Rev., 62, pp. 47–73 (1982).

K. Ohkawa et al., "Selective in Vitro and In Vivo Growth Inhibition Against Human Yolk Sac Tumor Cell Lines by Purified Antibody Against Human α–Fetoprotein Conjugated with Mitomycin C via Human Serum Albumin", Cancer Immunol. Immunother, 23, pp. 81–86 (1986).

C. W. Parker et al., "Enzymatic Activation and Trapping of Luminol–Substituted Peptides and Proteins. A Possible Means of Amplifying the Cytotoxicity of Anti–Tumor Antibodies", *Pro.Nat.Acad.Sci. USA*, 72(1), pp. 338–342 (1975).

G. W. Philpott et al., "Selective Iodination and Cytotoxicity of Tumor Cells with an Antibody–Enzyme Conjugate", *Surgery*, 74(1), pp. 51–58 (1973).

G. W. Philpott et al., "Selective Cytotoxicity of Hapten–Substituted Cells with an Antibody–Enzyme Conjugate", *The Journal of Immunology*, 111(3), pp. 921–929 (1973).

M. J. Robins et al., "Nucleic Acid Related Compounds. 16. Direct Fluorination of Uracil Nucleotides Using Trifluoromethyl Hypofluorite", *Can. J. Chem.*, 53, pp. 1302–1306 (1975).

G. F. Rowland et al., "Drug Localisation and Growth Inhibition Studies of Vindesine–Monoclonal Anti–CEA Conjugates in a human Tumour Xenograft", *Cancer Immunol* Immunother, 21, pp. 183–187 (1986).

F. Searle et al., "Antibody Carboxpeptidase G2 Conjugates as Anti–Tumor Agent", *Tumor Biology*, 6(4), pp. 355 (1985).

F. Searle et al., "The Potential of Carboxypeptidase G2 Antibody Conjugates as Anti–Tumor Agents. I. Preparation of Antihuman Chorionic Gonadotrophin–Carboxypeptidase G2 and Cytotoxicity of the Conjugate Against JAR Choriocarcinoma Cells In Vitro", *Br. J. Cancer*, 53, p. 377–384 (1986).

W. T. Shearer et al., "Cytotoxicity with Antibody–Glucose Oxidase Conjugates Specific for a Human Colonic Cancer and Carcinoembryonic Antigen", *Int. J. Cancer*, 14, pp. 539–547 (1974).

V. J. Stella et al., "Prodrugs Do They Have Advantages in Clinical Practice?", *Drugs*, 29, pp. 455–473 (1985).

W. A. Thomas, "Prodrugs", *Biochemical Society Transactions*, 14, pp. 383–387 (615th Meeting, Belfast 1986).

P. E. Thorpe et al., "The Preparation and Cytotoxic Properties of Antibody–Toxin Conjugates", *Immunological Rev.*, 62, pp. 119–158 (1982).

P.E. Thorpe, "Antibody Carriers of Cytotoxic Agents in Cancer Therapy: A Review", *Monoclonal Antibodies '84 Biological and Clinical Applications*, A. Pinchera et al (eds), pp. 475–506 (1985).

E. S. Vitetta et al., "Redesigning Nature's Posions to Create Anti–Tumor Reagents", *Science*, 238, pp. 1098–1104 (1987).

M. Y. Yeh et al., "Cell Surface Antigens of Human Melanoma Identified by Monoclonal Antibody", *Proc. Natl. Acad.Sci.USA*, 76(6), pp. 2927–2931 (1979).

M. Y. Yeh et al., "Clonal Variation in Expression of a Human Melanoma Antigen Defined by a Monoclonal Antibody", *J. Immunol.*, 126(4), pp. 1312–1317 (1981).

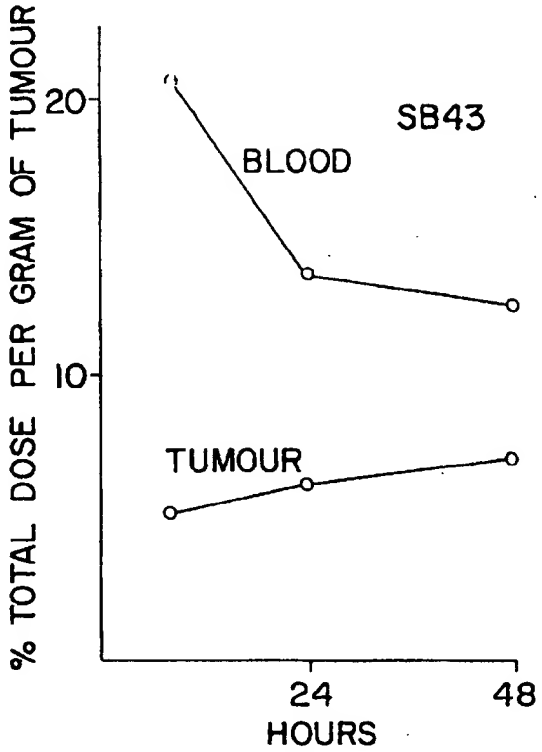

FIG. IA

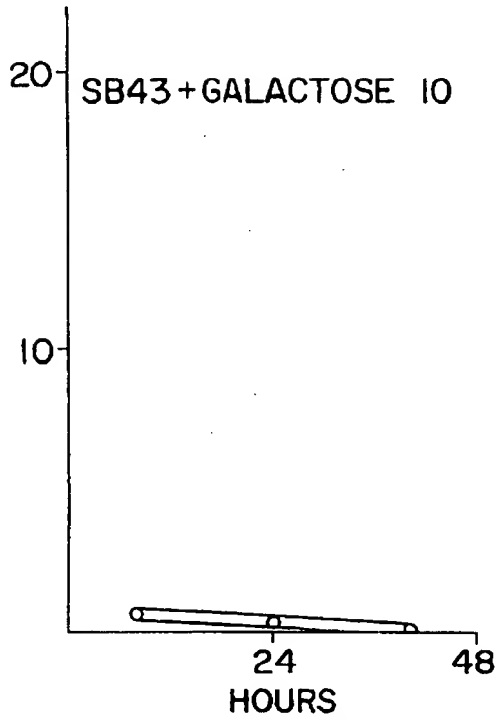

FIG. IB

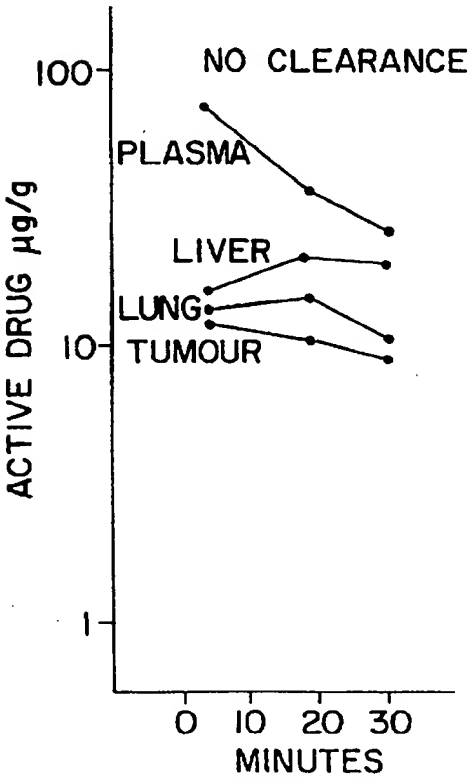

FIG. IC

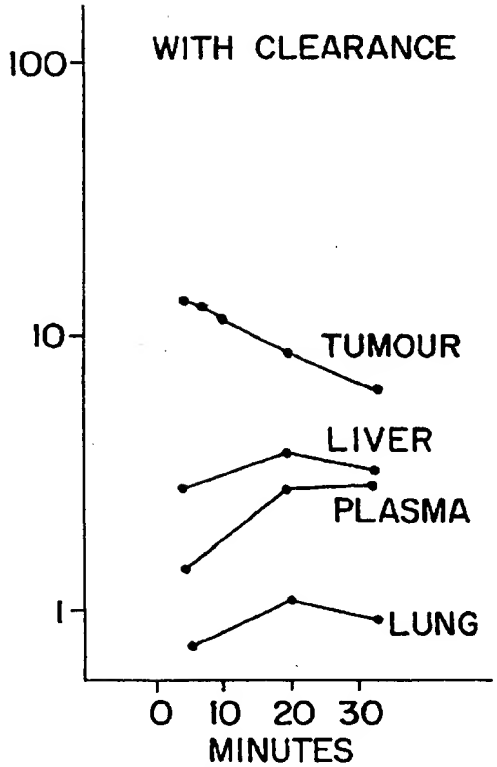

FIG. ID

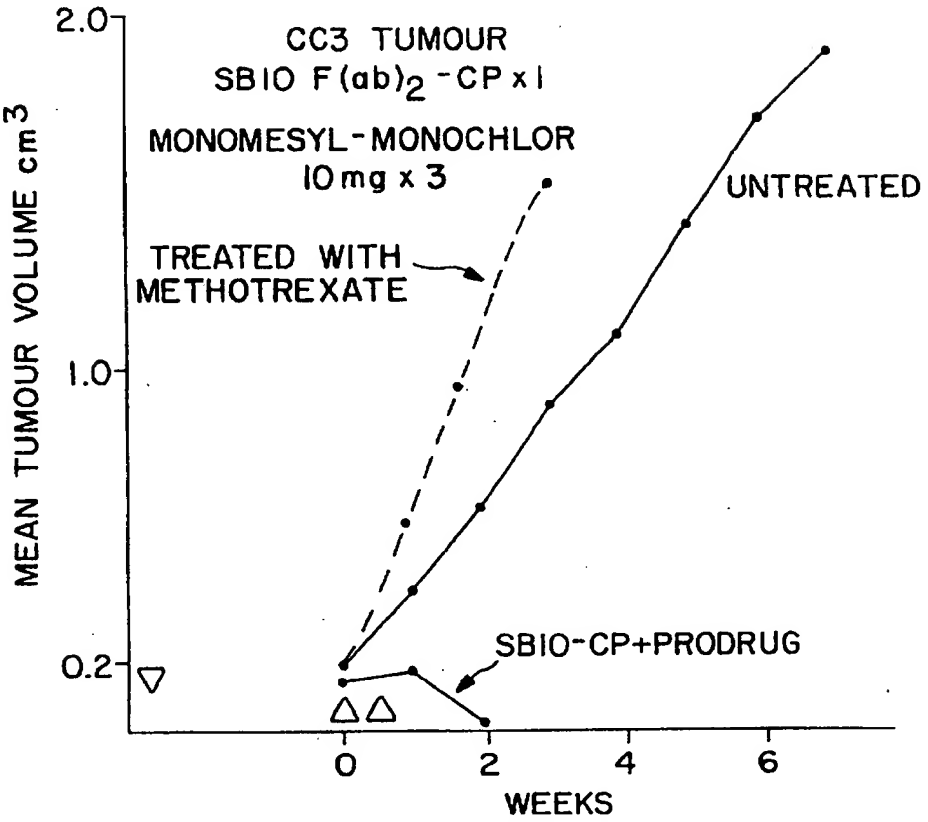
CC3 TUMOUR
SB10 F(ab)2 -CP x 1
MONOMESYL-MONOCHLOR
10 mg x 3
UNTREATED
TREATED WITH
METHOTREXATE
SB10-CP+PRODRUG
MEAN TUMOUR VOLUME cm3
2.0
1.0
0.2
0
2
4
6
WEEKS

FIG. 2A

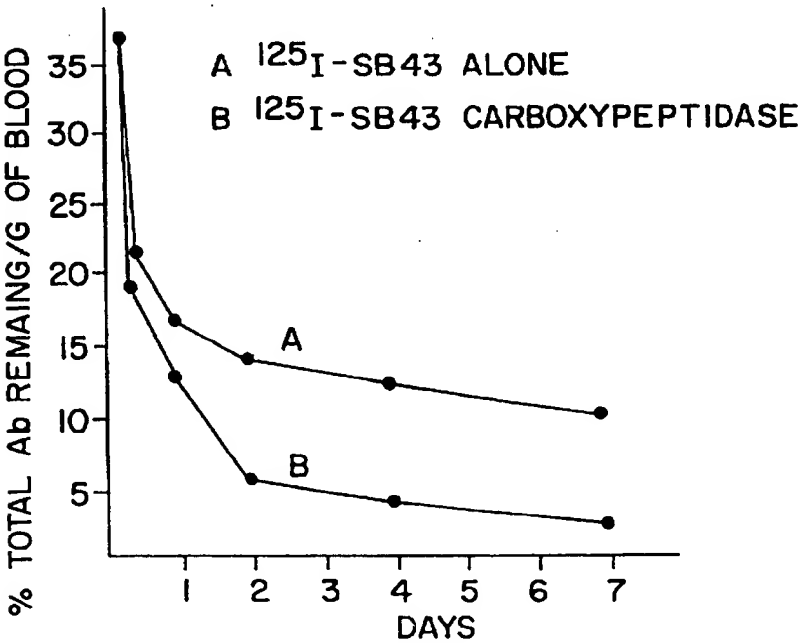
A 125I-SB43 ALONE
B 125I-SB43 CARBOXYPEPTIDASE
% TOTAL Ab REMAING/G OF BLOOD
35
30
25
20
15
10
5
A
B
1
2
3
4
5
6
7
DAYS

FIG. 2B

METHOD FOR THE TREATMENT OF TUMORS WITH CONJUGATED ANTIBODY A5B7 AND A PRODRUG

This is a Division of application Ser. No. 08/313,866 filed Sep. 28, 1994 which is a division of Ser. No. 07/642,301 filed Dec. 21, 1990 now abandoned.

This invention relates to methods and systems for the control of neoplastic cell growth and is particularly concerned with methods and systems involving the localisation of cytotoxic agents at tumour sites.

In our earlier Patent Application PCT/GB88/00181 we disclose a two-component system which comprises

(i) a first component (Component A–E) that is an antibody fragment capable of binding with a tumour associated antigen, the antibody fragment being bound to an enzyme capable of converting a prodrug into a cytotoxic drug; (ii) a second or final component (Component PD) that is a prodrug convertible under the influence of the enzyme to a cytotoxic drug (CD).

In our earlier PCT Patent Application and in this present Patent Application, the word 'tumour' is to be understood as referring to all forms of neoplastic cell growth including carcinomas, sarcomas, lymphomas and leukaemias.

Our existing system is used to target cytotoxic prodrugs to the site of neoplastic cell growth. However, although this two-component system is useful in the control of tumours, the amount of first component that localises per gram of tumour in humans may be less than 0.1% the total amount of first component administered. A substantial proportion of the non-localised first component including active enzyme remains in the circulating blood. It is therefore desirable to reduce the amount of this freely circulating antibody-enzyme first component before administration of the prodrug so as to limit the formation of active drug in blood from where it would be carried to normal tissues. A similar problem arises when the first component is prepared from whole antibody.

The present invention provides methods for the removal from blood of residual conjugates of the enzyme or for inactivation of the enzyme in the residual first component with whole antibody or antibody fragment or equivalent component after the first component has localised at tumour sites.

The present invention is concerned with reducing problems arising in the clinical use of our above-mentioned two-component system from the presence in the patient of non-localised first component and permits further extension to be made to the nature of the first component.

The present invention provides a three-component system, for use in association with one another, in the treatment of malignant diseases comprising: a first component which is a substance or conjugation of substances characterised by (a) one or more molecular configurations that are complementary to molecular configurations associated with malignant cells, such that the first component tends to localise selectively at sites of malignant cells and (b) additionally by one or more catalytic sites; a second component which is able to bind to such part of the first component so as to inactivate the catalytic site of the first component and/or accelerates the clearance of the first component from the blood when the first and second components are administered clinically; a third component which is a substrate for the catalytic site on the first component, one of the end products of the catalysis being a substance which is more cytotoxic to maligant cells than the third component.

The clinically most useful form of the first component is a conjugate of an antibody or fragment thereof and an enzyme while the clinically most useful form of the third component is a prodrug convertible under the influence of the enzyme activity of the first component into a cytotoxic compound. The antibody will desirably be one recognising and binding preferentially to a tumour associated antigen and it will be apparent to those skilled in the art how to match the antigen associated with the particular tumour to be treated with the antibody or fragment to be used in the first component, how to match the cytotoxic compound to the tumour to be treated and how to match the prodrug to the enzyme activity of the first component.

As described in our above-mentioned copending International Patent Application PCT/GB88/00181 the prodrug can be benzoic acid mustard glutamide that converts to benzoic acid mustard [p-(bis-2-chloroethyl)amino]benzoic acid under the influence of a carboxypeptidase. However, the principles of this invention are equally applicable to other prodrugs releasing benzoic acid mustard or analogues thereof or other cytotoxic drugs using enzymes appropriate to the removal from the prodrug of the structural feature distinguishing the prodrug from the cytotoxic drug.

When antibody is used in the first component, it can be whole antibody or one of the antibody fragments, e.g. $F(ab')_2$ or other fragment as described in our above-mentioned earlier filed International Patent Application. The function of the antibody in the first component is to assist in the localisation of the first component in the region of the tumour to be treated and this function can also be fulfilled by substances other than antibodies, e.g. hormones or growth factors that have affinity to other tumour-associated compounds.

In one embodiment of the system of the invention the first component is a conjugate of an antibody to a tumour-associated antigen or a fragment thereof that includes the antigen binding site of the antibody, said antibody or fragment thereof being conjugated directly, or indirectly through a linking component, to an enzyme or to an antibody or antibody fragment with catalytic functions. In this case the conjugation can be effected by chemical bonding or by splicing together nucleic acid sequences that code at least for one or more antigen binding sites and one or more catalytic sites and such other sequences as are necessary to retain the vector function of the molecule and the catalytic function of the peptide when the gene product of the reconstructed nucleic acid sequence is expressed by eukaryotic or prokaryotic cells.

In a further embodiment, the antibody in the first component is bivalent and formed by bonding together two univalent antibody fragments, or by recombinant DNA techniques, one fragment having affinity for a tumour marker substance, the other having affinity for an enzyme. In such a case the conjugate can be formed either in vitro prior to administration or in vivo by first administering the bivalent antibody, allowing time for it to localise at tumour sites and then administering the enzyme for capture by the second arm of the antibody localised at tumour sites.

The antibody of the first component may be a human immunoglobulin, or fragment thereof, having antigen binding site(s) of human origin or having antigen binding site(s) of non-human species.

Reichmann L, Clark M, Waldmann H, and Winter G (Reshaping human antibodies for therapy—Nature 332: 323–327, 1988) shows that by genetic engineering techniques the antigen binding sites of a rodent monoclonal can be incorporated into human immunoglobulin fragments so

that the immunogenicity of the molecule in the human subject is minimized. It has been shown that immunoglobulin-gene DNA can be manipulated so that the Fc portion of the antibody has been replaced with an active enzyme moiety (Neuberger M S, Williams G T, Fox R O—Nature 312: 604–608, 1984) and such genetically engineered constructs bearing one or more antigen binding sites and one or more enzyme active sites can be used in the present invention.

It has been observed that when monoclonal antibodies derived from one species are injected into another species the host antibody response may be (at least partially) directed at the idiotype of the injected monoclonal. (Rowe et al, IRCS Med Sci. 13: 936–7, 1985). Similarly, it is well-known that bacterial products, including enzymes, are immunogenic in mammalian species including man.

The present system will be most effective in man and suitable for repetitive use when the immunogenicity of a first component antibody-enzyme conjugate is minimised or if immune tolerance to such conjugates has been induced. This is likely to be achieved through genetic engineering methods since the production of monoclonals to specific antigens by human hybridomas has so far proved difficult to achieve consistently. It has been shown that the antigen binding site of a rodent monoclonal antibody can be incorporated into a human immunoglobulin framework (Reichmann et al, Nature 332: 323–327, 1988).

It has also been shown that antibodies can be produced which function as enzymes (Pollack S J et al, Science 234, 1570–1573, 1986) so the ultimate form of the antibody-enzyme conjugate may be a human immunoglobulin construct expressing one or more antigen binding sites characterised by peptide sequences of non-human origin and one or more catalytic sites.

With a 'humanised' antibody conjugated to a human enzyme or a non-human enzyme which has been rendered non-immunogenic in man, or a construct with both antigen binding and catalytic sites on a human immunoglobulin, the second component of our system will need to be directed at either the active site of the enzyme or at the idiotype of the antibody since a clearing antibody against the generality of human immunoglobulins would be unsuitable.

We have developed several different methods of removing what becomes the unwanted circulating first component after maximum localisation of the first component has occurred in the region of the tumour to be treated. The exact nature of the second component will depend upon the particular strategy to be used for removal of non-localised first component but the second component will always be one that either inactivates the catalytic site in the first component and/or accelerates its clearance from the blood.

According to one embodiment the second component is an antibody or fragment thereof having an affinity for an antigen binding site of an antibody of the first component or the active site of an enzyme of the first component or another constituent part of the first component.

According to a further embodiment the second component is one causing rapid loss of enzyme activity of the first component in plasma without incurring significant loss of enzyme activity from tumour sites.

According to a further embodiment the second component includes a sufficient number of covalently bound galactose residues or residues of other sugars such as lactose or mannose, so that it can bind enzyme in plasma but be removed together with the enzyme or antibody-enzyme conjugate from plasma by receptors for galactose or other sugars in the liver in a period of time such that the antibody does not, to any appreciable extent, enter the extravascular space of the tumour where it could inactivate tumour localised enzyme. In this case, galactose residues in the second component are either chemically added or exposed by removing terminal sialic acid residues.

Terminal sialic residues play a role in maintaining the presence of glycoproteins in the blood. Removal of terminal sialic acid by neuraminidase exposes proximal sugar residues such as galactose. Desialylated proteins are rapidly removed from the blood by receptors in liver and possibly other sites. (Morell et al, J.Biol. Chem. 246: 1461–1467, 1971).

Asialo human chorionic gonadotrophin was prepared by digesting 1 mg of a glycoprotein in 1 ml of 0.05M sodium acetate buffer, pH 5.6, containing 0.15 M NaCl with 20 μg of neuraminidase (Sigma Type II from Vibro cholerae) at 37° for 30 minutes. The neuraminidase was then removed. Sialo and Asialo preparations were compared for clearance in A2G mice. T ½ for the sialyted hCG was in excess of 24 hours but T ½ for the desialyted form was <5 min.

A further embodiment of the invention is one wherein the first component is an antibody enzyme conjugate modified by addition of, or exposure of, additional galactose or other sugar residues, and is for administration in conjunction with an agent, such as asialofetuin, which has greater affinity for the corresponding sugar receptors which are involved in removing galactosylated or similar proteins from the blood, the asialofetuin blockade being maintained until satisfactory levels of conjugate have been attained in the tumour, then allowing the concentration of the conjugate in the plasma to fall before administering the third component.

This embodiment requires the addition of galactose residues to the antibody-enzyme conjugate by methods similar to those described for galactosylating the clearing antibody. Before administering the galactosylated conjugate the galactose receptors are blocked by an agent which binds more avidly to those receptors than the galactosylated conjugate. This results in maintenance of a high level of conjugate in the plasma until galactose receptors are again free to take up the conjugate.

Asialofetuin is a substance known to bind strongly to galactose receptors but other less immunogenic substances could be identified or developed for the same purpose.

A further embodiment of the invention is one wherein the second component is conjugated to a macromolecule such as a dextran, liposome, albumin microsphere or macroglobulin with a molecular weight in excess of 500,000 Daltons or a biodegradable particle such as a blood group 0 erythrocyte so that the second component is restrained from leaving the vascular compartment by virtue of the size of the conjugate.

A further embodiment of the invention is one wherein the second component is an antigen, hapten or protein construct bearing an epitope capable of binding with the first component to form complexes having accelerated clearance from plasma.

In a still futher modification of the system, the first component is covalently linked to biotin or derivatives of biotin, and the second component then comprises the biotin-binding glycoprotein avidin found in egg white, or streptavidin, itself optionally covalently linked to galactose.

Biotin may be conjugated to antibody or fragment thereof by reaction with a 10 molar excess of sulphosuccinimidyl 6-(biotinamido)hexanoate at pH 8.5 at 4° C. for 16 hours. The product is purified by chromatography on Sephadex G-25.

In all these cases, except for the procedure involving asialofetuin, the second component may be conjugated with

a macromolecule or biologically degradable particle such that the additional component does not, to any appreciable extent, escape from the vascular compartment. The macromolecule may be optionally galactosylated.

Macromolecules to limit clearing component escaping from the vascular compartment are likely to be in excess of 500,000 Daltons and include carbohydrates such as dextrans, lipids as in liposomes or proteins such as in albumin microspheres or a macroglobulin. An example of a biodegradable particle for this purpose is an erythrocyte of blood group 0.

As an alternative to basing the first component on an antibody it may be based on a hormone or growth factor or substance other than an antibody and for which receptors capable of binding that substance exist on a tumour.

Tumours may express receptors for growth factor hormones and other metabolites such that these can be used as target sites for selective delivery. The corresponding growth factor, hormone, metabolite or genetic construct might then be used as the vector to carry an enzyme to tumour sites in a comparable fashion to antibody. There are literature examples of radiolabelled hormones, growth factors and metabolites localising in tumours (Krenning et al, Lancet i 242–244, 1989 (Somatostatin); Hattner et al, Am. J. Roentgenol. 143: 373–374, 1984) but in none of these were the vectors used to convey enzyme to tumour sites.

The enzyme part of the first component can be of human or non-human origin. The advantage of using an enzyme of human origin lies in avoiding or minimising the immunogenic effect of an enzyme of non-human origin. The disadvantage of an enzyme of human origin is the probability that the presence of enzyme in human tissues will activate the prodrug, thus releasing active drug at the non-tumour sites. However, it may be possible to identify certain human enzymes which are so distributed that this activation would not cause a serious problem. Also, inactivation of such enzymes in tissues might be achieved by using high affinity anti-enzyme antibody fragments which would be rapidly cleared from the plasma before giving the first component conjugate. Where the human enzyme is normally present in the plasma, this would activate prodrug in the plasma which would be highly disadvantageous and be liable to cause general toxic effects. Administration of an appropriately selected antibody or antibodies or fragments directed at the enzyme in the conjugate would however also have the effect of inactivating free, naturally recurring enzyme of the same type in the plasma. In the case of human phosphatases there are several different forms produced in different tissues but there is little evidence of specificity for substrates. There is also evidence that antibodies directed at one isotype of alkaline phosphatase may bind to other isotypes.

The immunogenicity of an enzyme of non-human origin may be reduced by modification of its amino acid sequence.

In order to render the antibody-enzyme conjugate less immunogenic, it can be modified by conjugation to polyethylene glycol or other polymers, e.g. by reaction with the cyanuric chloride derivative of methoxypolyethylene-glycol 5000. The resulting material may be employed directly, or may be pre-injected to render the host tolerant to further injections of the native conjugate. Reaction with synthetic copolymers of D-glutamine acid and d-lysine or with tripeptidyl-modified organic polymers comprising alternate D-glutamic acid and D-lysine on the exterior ends of the side chains can be predicted to depress the immunogenicity of the conjugate. See, for example, Abuchowsky A., van Es T., Palezuk N C, Davis F F—J. Biol. Chem. 252: (11), 3578–81, 1977, or Kawamura K, Igarishji, T, Fujii T., Kamasaki J., Wada, H., Kishimoto, S. Int. Arch. Allergy appl. Immunol. 76: 324–330, 1985.

To minimise clinical problems arising from the use of immunogenic antibody enzyme conjugates and immunogenic antibodies or avidin-like constructs, it is desirable to minimise or delay the production of host antibodies to xenospecific proteins by using immunosuppressive agents such as cyclosporin, cyclophosphamide, methotrexate, azathioprine etc., in order to provide sufficient time for the delivery of repeated treatments.

The ability of cyclosporin to prevent antimurine antibody responses by rabbits and in patients has been demonstrated. See, for example, Ledermann, J A. Begent, R H J. Bagshawe, K D. Br. J. Cancer, 58: 562–566, 1988, or Ledermann, J A. Begent, R H J. Riggs, S J. Searle, F. Glaser, M G, Green, A J. Dale, R G. Br. J. Cancer 58: 654–657, 1988.

In certain clinical conditions, it can be advantageous for the first component to be conjugated to a signal producing molecule such as a radioisotope suitable for scintigraphic imaging by gamma camera so as to confirm localisation of the first component at tumour sites.

Radiolabelling can be achieved with ^{125}I or ^{131}I with standard methods either using chloramine T (Greenwood F, Hunter W, Glover J S, Biochem. J. 89: 114–123, 1963, Fraker P J, Speck J C, Biochem. Biophys. Res. Comm. 80: 849–857, 1978), but other methods of iodination or radiolabelling with other isotopes such as indium or technetium can also be used. Such radiolabelled conjugates are generally used in clinical practice in amounts required for radioimmunolocalisation by immunoscintigraphy and would generally form only a small part of the administered conjugate.

Modern methods of analysis may be used in conjunction with a radiolabelled fraction of the conjugate to determine the concentration of the conjugate at target sites and non-target sites and thus help determine the optimum time for administration of the prodrug. (Riggs et al, Int. J. Cancer Supp. 2, 95–98, 1988, Dewhurst et al, (Abstract) Br. J. Cancer 1988).

The system of the present invention can include more than one type of first component and/or more than one type of second component and/or more than one type of third component. Heterogeneity in expression of target antigens and receptors on cells in tumours may require the use of more than one vector to carry enzyme to tumour sites. Multiple vectors may permit greater or more economical delivery of enzyme to tumour sites. It may also be advantageous to use more than one type of prodrug to generate a state equivalent to multidrug chemotherapy so as to reduce the risks of drug resistance and this in turn may require the use in treatment of more than one type of enzyme. These variations may in time require the use of more than one second component to achieve the required clearing of enzymes from plasma and other non-tumour sites.

The three components forming the system of the present invention are designed to be used in association with one another in a method of treatment of the human or animal body by therapy.

It is specifically designed for use in a method for the treatment of malignant diseases including carcinomas, sarcomas, lymphomas and leukaemias which comprises administering to a host in need of such treatment an effective amount of a system.

In such a method, the first component is administered first, the second component is administered subsequent to the first component after a time interval such that the first component has selectively localised at the site of malignant

cells and the third component is administered subsequent to the second component after a time interval such that the concentration of the first component in the blood has reduced from its peak value.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A and FIG 1B show blood and tumour levels up to 48 hours after injection of monoclonal SB43 (anti-carboxypeptidase). A, native form; B, galactosylated form.

FIG. 1C and FIG. 1D show the levels of active drug in various tissues in mice which have not received SB43-Gal 10 clearing antibody with those that had.

FIG. 2A shows the result of administering SB10 $F(ab')_2$-CP (50 units CP) intravenously to 6 CC3 bearing nude mice, followed by the first of three injections of monomesyl monochloro benzoic acid mustard prodrug 4-[(2-chloroethyl)mesylamino]benzoic acid glutamide.

FIG. 2B shows accelerated clearance of 20 μg monoclonal ^{125}I-SB43 anticarboxypeptidase from the blood of A2G mice when 77 μg of the corresponding antigen, carboxypeptidase G2, was administered 1 hour later compared with controls which did not receive the antigen.

The following Examples are given to illustrate various aspects of the invention.

EXAMPLE 1

This is to illustrate the inactivation of an active enzyme site by an antibody.

A monoclonal antibody (SB43) was produced by conventional methods following immunisation of the lymphocyte donor mouse with carboxypeptidase G_2. Microtitre plates were coated with three units per well of carboxypeptidase G_2 and incubated with supernatants from the hybridoma culture, and it was found that ^{125} Iodine labelled rabbit anti-mouse antibody bound to the coated wells with a 50% binding titre at a dilution of the supernatant of 1:800 in buffered solution. Assay of enzyme activity was assessed after 24 hours incubation at 37° C. in buffer containing a 1000-fold dilution of the antibody (hybridoma supernatant). Enzyme incubated with buffer alone for 24 hours retained most of its capacity to cleave methotrexate as shown by optical density measurements (54.1 carboxypeptidase units/ml initially falling to 40 units/ml activity after 24 hours). In the wells containing the antibody (hybridoma supernatant) the activity was reduced to 13.0 carboxypeptidase units/ml. The antibody alone had no effect on the optical density of methotrexate. These experiments show that the enzyme active site on the carboxypeptidase can be substantially inactivated by an antibody raised against the enzyme. Monoclonal antibodies to carboxypeptidase G_2, raised by the technique described above will only have a similar enzyme inhibiting property if they are directed at epitopes in or close to the active site of the enzyme.

EXAMPLE 2

Evidence for localisation of antibody-enzyme conjugate at tumour sites

1. 4 nude mice bearing LS174T human colon cancer xenografts on their L flanks were injected with A5B7 $(Fab')_2$ monoclonal antibody directed at carcinoembryonic antigen conjugated to carboxypeptidase G2 and labelled with ^{125}I. The hybridoma which produces monoclonal antibody A5B7 has been deposited on Jul. 14, 1993 pursuant to the Budapest Treaty with the European Collection of Animal Cell Cultures (ECACC) and has been given the accession number: ECACC 93071411. The address of the European Collection of Animal Cell Cultures is: ECACC, Public Health Laboratory Service, Center for Applied Microbiology and Research, Porton Down, Salisbury, Witshire SP4OJG, United Kingdom. An immunoscintigraph taken after 48 hours confirms localisation of the conjugate at the tumour sites.

Similar results were obtained using the following conjugates:

2. A5B7 intact IgG—carboxypeptidase

3. A5B7-$F(ab')_2$—nitroreductase

4. SB10 (antiHCG)—$F(ab')_2$ —carboxypeptidase

2. METHODS OF CONJUGATION OF ANTIBODY TO ENZYME

Conjugation of IgG or $F(ab')_2$ with carboxypeptidase was accomplished by mixing a maleimide derivative of the enzyme with a thiolated antibody.

1) Thiolation with S-acetylthioglycolic acid N-hydroxysuccinimide ester (SATA)

IgG or $F(ab')_2$ in 0.1 M sodium phosphate buffer, pH 7.6, (containing 372 mg of EDTA/liter) at 1–2 mg/ml was treated with a 15 molar excess of SATA (made up 20 mg/ml in DMF) and left at about 20° C. for approximately 2 hours. The thiolated antibody was then passed down a column of Sephadex G-25 to remove excess SATA. The thiol was deacetylated by adding 0.1 volumes of 3.5% hydroxylamine, pH 7.5, prepared by adding disodium hydrogen phosphate to an aqueous solution of hydroxylamine hydrochloride.

2) Thiolation with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)

IgG or $F(ab')_2$ in 0.2M sodium phosphate buffer, pH 8.6, at a concentration of 4 mg/ml was treated with a 15 molar excess of SPDP in ethanol and left at r.t. for 1 hour. Excess SPDP was removed on a column of Sephadex G-25 equilibrated in 0.1M sodium acetate buffer, pH 4.5. The pyridyldisulphide group was then reduced for 30 minutes with 50 μl/ml of 100 mM dithiothreitol and excess reducing agent removed by Sephadex G-25 gel filtration.

3) Derivatisation of carboxypeptidase

Carboxypeptidase in 0.1M sodium phosphate buffer, pH 7.6 (containing 372 mg EDTA/liter) at 2 mg/2.5 ml was treated for 3 hours with a 15 molar excess of succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) dissolved in THF. The excess SMPB was removed by gel filtration on Sephadex G-25.

4) Conjugation

The derivatised enzyme was mixed with an equimolar amount of thiolated antibody and the progress of the conjugation monitored by gel filtration. When no further reaction was judged to take place the mixture was concentrated and the conjugate purified by gel filtration. Typical enzyme activities obtained were 150–200 units/mg of conjugate.

EXAMPLE 3

Evidence that SB43 binds to carboxypeptidase G2 in vitro.

Microtitre wells were coated with carboxypeptidase G2 and monoclonal antibodies SB43 (raised to carboxypeptidase G2) and SB10 (raised to human chorionic gonadotrophin) were added in dilution and incubated before aspiration. ^{125}I anti-mouse IgG was then added and incu-

bated for 30 minutes followed by aspiration and washing. The wells were cut out and counted in a gamma counter. The results set out below show no significant binding of SB10 to the carboxypeptidase coated wells but all dilutions of SB43 used showed high counts indicating binding to the carboxypeptidase. SB43 modified by addition of galactose moieties was included a similar dilution and showed similar binding to unmodified SB43.

The microtitre plate was coated with 0.1 microg. CPG_2 per well and incubated overnight with SB43. The ^{125}I-mouse IgG was then introduced, the plate incubated for 1 hour, washed and radio-counted.

SAMPLE	TIME	COUNTS(1)	CPM(1)	% CV	
1	30	1092	2171.4	3.1	(Negative Control-SB10-Anti-hCG)
2	30	805	1588.6	3.6	(Negative Control-SB10-Anti-hCG)
3	30	16428	33278.0	.8	SB43 × 20
4	30	23339	47302.9	.7	SB43 × 20
5	30	22020	44679.3	.7	SB43 × 100
6	30	22096	44833.8	.7	SB43 × 100
7	30	8437	16873.4	1.1	SB43 × 1000
8	30	7671	15336.8	1.1	SB43 × 1000
9	30	15411	31052.2	.8	SB43-Gal-10 × 20
10	30	15418	31066.3	.8	SB43-Gal-10 × 20

EXAMPLE 4

Evidence that SB43 inactivates/clears Ab-E-conjugate in vivo from plasma

The level of carboxypeptidase G2 activity in plasma can be monitored by observing the hydrolytic cleavage of methotrexate, a folic acid analogue, to pteroates and L-glutamate. When a conjugate of A5B7-$F(ab')_2$-carboxypeptidase G2 (25 enzyme units) was injected intravenously and plasma samples obtained 20 hours later significant hydrolysis of methotrexate was observed equivalent to 1.12 to 1.45 enzyme units/ml as shown by the steps of the spectrophotometric print-out.

Mice which were injected with galactosylated anti-carboxypeptidase (SB43-Gal 10) 19 hours after A5B7F(ab')$_2$-CPG2 and plasma taken 5 minutes and 15 minutes later caused no significant hydrolysis of methotrexate showing that the enzyme had been inactivated and/or cleared from the plasma.

EXAMPLE 5

Biotinylation of antibody-enzyme conjugate

Carboxypeptidase G2 (44.4 mg) in 0.05 M sodium bicarbonate buffer, pH 8.5 (1.5 ml) was mixed with sulphosuccinimidyl 1-6-(biotinamide) hexanoate (292 µg in 73 µl of buffer and left at room temperature for 3 hours. The enzyme was then separated on Sephadex G-25 equilibrated in 0.15M sodium phosphate buffer pH 7.6, containing 372 mg EDTA/liter and the volume adjusted to 2.5 ml. The biotinylated enzyme was then treated for 3 hours with a 15 molar excess of succinimidyl 4-(p-maleimido phenyl)butyrate (SMPB) dissolved in tetrahydrofuran and the excess SMPB removed by gel filtration on Sephadex G25. The derivatised enzyme was then conjugated to thiolated $F(ab')_2$ fragment of the A5B7 antibody as described in Example 2.

Affinity purified avidin was used as obtained from Sigma Ltd., 10–15 units/mg protein. Mice received 20 µg of biotinylated A5B7-carboxypeptidase G2 conjugate followed after one hour by avidin in the dose range 20–500 µg. Rapid clearance of the enzyme activity in plasma was observed comparable to that observed with SB43 monoclonal antibody in Example 4.

EXAMPLE 6

IgG class immunoglobulins carrying different specificities on their two binding sites can be made by a fusion technique employing hybridomas producing different antibodies (Milstein C & Cuello A C. Nature 305: 537-540, 1983; Sfaerz U D & Bevan M J. Proc. Nat. Acad. Sci. USA 83: 1453–1457, 1986) or by chemical conjugation of univalent preparations of each of the antibodies required as used here. $F(ab')_2$ fragments of monoclonals SB10 (anti-human chorionic gonadotrophin (anti-hCG)) and A5B7 (anti-carcinoembryonic antigen, (anti-CEA)) were reduced in the presence of arsenite. $F(ab')_2$ fragment (20 mg) in 0.1M sodium phosphate buffer pH 7.6 (10 ml) was mixed with sodium arsenite (12.4 mg) EDTA (3.72 mg) and 2-mercaptoethylamine (1.13 mg) and left at room temperature. Solid 5,5'-dithio-bis-(2-nitrobenzoic acid) (19.8 mg) was added and the mixture left at about 20° C. for 18 hours. The thionitrobenzoate modified Fab' (TNB derivative) was purified by gel filtration on Sephadex G-25 with a yield of approximately 70% based on protein recovery.

The TNB derivative of anti-CEA (4.8 mg) in 5 ml of 0.1M sodium phosphate buffer, pH 6.8, containing 1 mM EDTA was treated for 30 minutes with mercaptoethylamine to give a final concentration of 10 mM. The reduced TNB-anti-CEA Fab' was then purified by gel filtration on Sephadex G-25 equilibrated in 0.1M sodium phosphate buffer pH 7.0, containing 1 mM EDTA. The reduced TNB-anti-CEA Fab' was then incubated with 4.9 mg (5 ml) of TNB derivative of anti-hCG for 16 hours and the formation of bispecific antibody monitored by gel filtration on a Superose S-12 column (Pharmacia). The yield was 20% based on protein after purification of the bispecific antibody on the Superose S-12 column. The ability of this ^{125}I labelled biospecific antibody to function in vivo and bind to its corresponding antigen was demonstrated by injection into nude mice bearing either CEA producing LS174T tumours or hCG producing CC3 tumours. At 20 hours post-injection mean tumour to organ ratios were:

	anti CEA/anti hCG	non-specific $F(ab')_2$
blood	2.9	0.6
liver	3.9	1.9
kidney	1.8	1.2
lung	3.2	1.2
spleen	6	3.0
colon	9	5.3

(Conjugation of A5B7 and SB43 (anticarboxypeptidase) has not yet been performed but above experiment demonstrates retention of binding site function).

EXAMPLE 7

Method for galactosylation

Cyanomethyl 2,3,4,6-tetra-0-acetyl-1-thio-b-D) galactopyranoside (400 mg) in anhydrous methanol (10 ml) was treated with 5.4 mg of sodium methoxide in 1 ml of anhydrous methanol at about 20° C. for 48 hours. A stock solution of IgG in 0.25M sodium borate buffer, pH 8.5 at 1.3 mg/ml was prepared. Since the number of galactose residues

conjugated to IgG was not determined, a unitage was adopted corresponding to the number of microliters of the activated galactose derivative added to 200 μg of IgG at a concentration of 1.3 mg/ml.

Aliquots of the activated galactose derivative (e.g. 300, 80, 40, 10, 5 and 2 μl) were dispensed into 3 ml glass ampoules and evaporated to a glassy residue in a stream of nitrogen under vacuum. 200 micrograms of IgG (153 μl of stock solution) were added to each aliquot and mixed until the residue was dissolved. After 2 hours at about 20° C. the solution was dialysed against 3 changes of PBS (phosphate buffer saline). Tests were performed to determine what level of galactosylation gave the most effective results. It was found that, in terms of the unitage defined above, 10 μl of the activated glactose derivative added to 200 μg of IgG gave the most satisfactory results. FIG. 1A and FIG. 1B shows blood and tumour levels up to 48 hours after injection of monoclonal SB43 (anti-carboxypeptidase). A, native form; B, galactosylated form. Further studies were performed, each in groups of 4 mice, bearing LS174T tumours and receiving A5B7 F(ab')2-CP (carboxypeptidase G2) conjugate followed after 24 hours by SB43 (anti-carboxypeptidase) galactosylated to the 10 μl level (as defined previously) or with saline as control followed one hour later by the bis-chloro benzoic acid mustard prodrug, 4-[bis-(2-chloroethyl)amino]-benzoic acid glutamide. Mice were killed at intervals following administration of the prodrug, the tissues extracted and prodrug and active drug levels were measured by HPLC methods.

FIG. 1C shows the levels of active drug in various tissues in mice which had not received SB43-Gal 10 clearing antibody with those that had. In the absence of SB43-Gal 10 clearing antibody, levels of active drug were significantly lower than those found in liver and lung but in animals receiving the SB43-Gal 10 clearing antibody tumour levels were higher than in any other tissue.

As part of the same experiment two groups of mice, one with and one without SB43-Gal 10, were killed without receiving the prodrug. The tissues were extracted and tested for ability to convert prodrug to active drug in vitro, The results are shown in the Table and expressed as percentage of injected dose of carboxypeptidase per gram of tissue.

	Carboxypeptidase G2% i.v. dose per gram of tissue at 48 hours		
In vivo admin	Tumour	Plasma	T/P
Ab-CPG2	8.1(+0.69)	0.22	36
AB-CPG2 + Gal 10 antiCPG2 24 hours later	7.2(+1.42)	0.026	277

EXAMPLE 8

Where the antigen corresponding to an intravenously administered antibody is present in the blood, antigen-antibody complexes form and these accelerate clearance of the antibody from the circulation into the reticuloendothelial cells. Accelerated clearance of anti-hCG antibodies W14 and SB10 occurs when these are injected into nude mice bearing CC3 hCG secreting tumours when compared with A5B7 anti-CEA antibody in LS174T bearing mice which express CEA on LS174T cell membranes but do not secrete CEA into the blood.

FIG. 2A shows the result of administering SB10 $F(ab')_2$-CP (50 units CP) intravenously to 6 CC3 bearing nude mice, followed by the first of three injections (10 mg each) of the monomesyl monochloro benzoic acid mustard prodrug 4-[(2-chloroethyl)mesylamino]benzoic acid glutamide, the second given at 56 hours and the third at 72 hours. After 2 weeks the tumour was no longer detectable and the mice remain tumour free at 12 weeks. The growth of CC3 tumours in 6 untreated mice is also shown.

Attempts to introduce the prodrug into LS174T bearing mice before 120 hours after administration of A5B7 $F(ab')_2$-CP 50 units resulted in death of the animals and this was shown to be due to persisting enzyme activity in the blood.

FIG. 2B shows accelerated clearance of 20 μg monoclonal ^{125}I-SB43 anticarboxypeptidase from the blood of A2G mice when 77 μg of the corresponding antigen, carboxypeptidase G2, was administered 1 hour later compared with controls which did not receive the antigen.

These data indicate that accelerated clearance of an administered antibody can be achieved by administration of a substance expressing the epitope corresponding to the binding site of the antibody.

EXAMPLE 9

Conjugation of TCK9 human albumin microspheres to SB43

1 mg of TCK9 human polyalbumin microspheres were derivatised with a 12.5M excess of sulpho-MBS (based on monomeric unit of 66 Kd) in a total of 1 ml phosphate buffer pH 7.8 for 2 hours at about 20° C. The mixture was centrifuged at 3000 rpm for 3 minutes and resuspended in 1 ml of buffer, and rewashed once more. 1.5 mg of ^{125}I labelled SB43 was thiolated by 20M excess of SPDP, according to manufacturers (Pharmacia) instructions, at about 20° C. The derivatised microspheres were centrifuged at 3000 rpm for 3 minutes, and then resuspended in the thiolated SB43 solution, the conjugation was carried out by incubating the mixture at 4° C. for 72 hours.

The antibody polyalbumin conjugate was separated from the reaction mixture by centrifuging at 13000 rpm (MSE Micro centaur) for 2 minutes; the pellet was resuspended in 250 μl of sterile saline for use.

EXAMPLE 10

Asialofetuin was given intravenously at time zero and again at 120 minutes to mice bearing LS174T xenografts. ^{125}I-A5B7-galactosylated to 10 units was administered at time +5 minutes. Mice were killed at intervals and tissues excised and radioactivity levels counted. At 24 hours the tumour to blood ratio was 27.8:1 and the tumour to liver ratio 4.2:1. All other tissues showed even more favourable ratios. It should be recognised that whereas enzyme taken up by liver is rapidly inactivated, radioactivity persists in the organ.

We claim:

1. A method of treatment of tumors to which antibody A5B7 binds, said method comprising administering to a host in need of such treatment a system which comprises:

i) a conjugate comprising

a) antibody A5B7 ; linked to

b) an enzyme selected from the group consisting of carboxypeptidase G2 (CPG2) and nitroreductase; and,

ii) a prodrug convertible under the influence of the conjugate into a cytotoxic drug.

2. A method of treatment of tumors to which antibody A5B7 binds, said method comprising administering to a host in need of such treatment a system which comprises:

i) a conjugate of A5B7 antibody and carboxypeptidase G2 enzyme; and,

ii) a prodrug convertible under the influence of the conjugate into a cytotoxic drug.

3. A method of treatment of tumors to which antibody A5B7 binds, said method comprising administering to a host in need of such treatment a system which comprises:

i) a conjugate comprising

a) antibody A5B7 ; linked to

b) an enzyme selected from the group consisting of carboxypeptidase G2 (CPG2) and nitroreductase; and,

ii) a mustard prodrug convertible under the influence of the conjugate into a cytotoxic drug.

4. A method of treatment of tumors to which antibody A5B7 binds, said method comprising administering to a host in need of such treatment a system which comprises:

i) a conjugate of A5B7 antibody and carboxypeptidase G2 enzyme; and,

ii) a mustard prodrug convertible under the influence of the conjugate into a cytotoxic drug.

5. A method of treatment of tumors to which antibody A5B7 binds, said method comprising administering to a host in need of such treatment a system which comprises:

i) a humanized A5B7 antibody linked to a member of the group consisting of a human enzyme, a non-human enzyme rendered non-immunogenic in man and a humanized catalytic antibody; and,

ii) a prodrug convertible under the influence of (i) into a cytotoxic drug.

6. The method according to claim 1 wherein the antibody lacks an Fc portion.

7. The method according to claim 1 wherein the antibody is a F (ab')2 structure.

8. The method according to claim 1 wherein the antibody is linked to the enzyme via a continuous polypeptide linkage.

9. The method according to claim 1 wherein the antibody is humanized.

10. The method according to claim 2 wherein the antibody lacks an Fc portion.

11. The method according to claim 2 wherein the antibody is a F (ab')2 structure.

12. The method according to claim 2 wherein the antibody is linked to the enzyme via a continuous polypeptide linkage.

13. The method according to claim 2 wherein the antibody is humanized.

14. The method according to claim 3 wherein the antibody lacks an Fc portion.

15. The method according to claim 3 wherein the antibody is a F(ab')2 structure.

16. The method according to claim 3 wherein the antibody is linked to the enzyme via a continuous polypeptide linkage.

17. The method according to claim 3 wherein the antibody is humanized.

18. The method according to claim 4 wherein the antibody lacks an Fc portion.

19. The method according to claim 4 wherein the antibody is a F(ab')2 structure.

20. The method according to claim 4 wherein the antibody is linked to the enzyme via a continuous polypeptide linkage.

21. The method according to claim 4 wherein the antibody is humanized.

22. The method according to claim 5 wherein the antibody lacks an Fc portion.

23. The method according to claim 5 wherein the antibody is a F(ab')2 structure.

24. The method according to claim 5 wherein the antibody is linked to the member via a continuous polypeptide linkage.

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(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF SOUTHAMPTON [GB/GB]; Highfield, Southampton SO9 5NH (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)* : GLENNIE, Martin, John [GB/GB]; The Tenovus Research Laboratory, Southampton General Hospital, Tremona Road, Southampton SO9 4XY (GB).

(74) Agents: WOODS, Geoffrey, Corlett et al.; J.A. Kemp & Co, 14 South Square, Grays Inn, London WC1R 5EU (GB).

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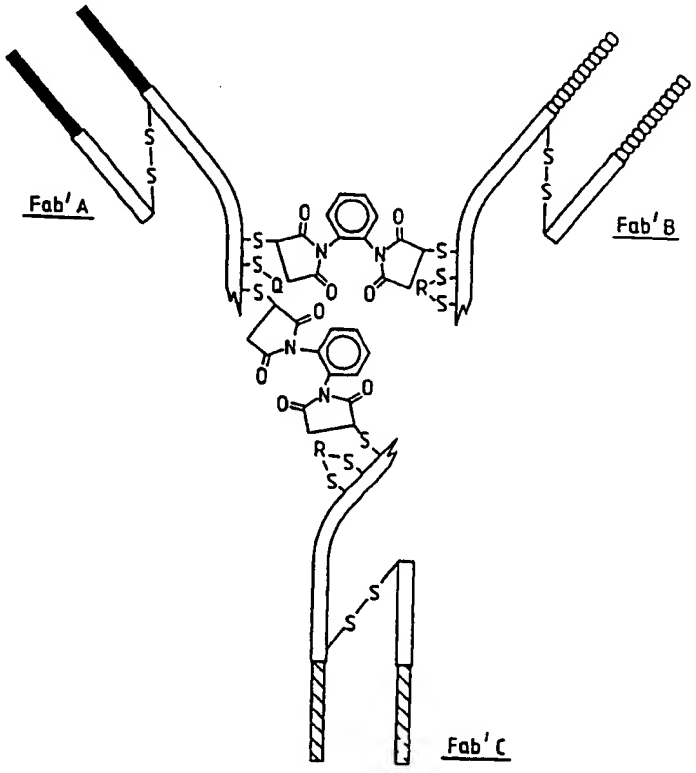
(54) Title: BI-OR TRISPECIFIC $(FAB)_3$ OR $(FAB)_4$ CONJUGATES

(57) Abstract

Novel trimeric and tetrameric antibodies are disclosed, including bispecific and trispecific $F(ab)_3$ and $F(ab)_4$ antibodies. A simple and efficient method is described for the production of pure $F(ab'\gamma)_3$ antibodies, in which the individual antibody Fab' fragments are joined via stable thioether linkages. Hybrid molecules were constructed from mouse monoclonal antibodies with specificities for targetting cytotoxic effectors (human peripheral blood T cells) against ^{51}Cr-labelled chicken red blood cells. Fab' fragments from two of the chosen antibodies were first coupled via their hinge-region SH groups using o-phenylenedimaleimide (oPDM), this bispecific fragment was then linked, again via the hinge region using oPDM, to a third Fab' fragment.



TRISPECIFIC $F(ab')_3$ ABC

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(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF SOUTHAMPTON [GB/GB]; Highfield, Southampton SO9 5NH (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)* : GLENNIE, Martin, John [GB/GB]; The Tenovus Research Laboratory, Southampton General Hospital, Tremona Road, Southampton SO9 4XY (GB).

(74) Agents: WOODS, Geoffrey, Corlett et al.; J.A. Kemp & Co, 14 South Square, Grays Inn, London WC1R 5EU (GB).

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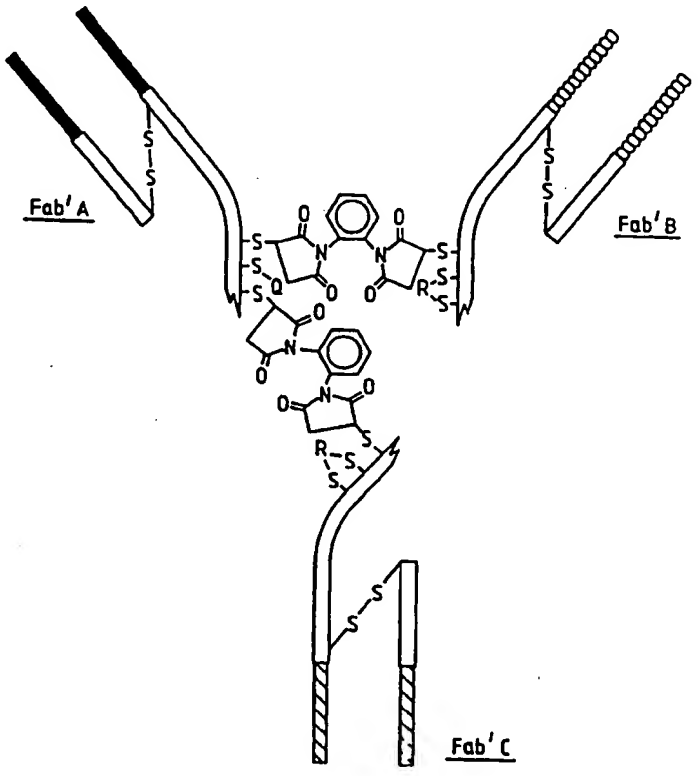
(54) Title: BI-OR TRISPECIFIC $(FAB)_3$ OR $(FAB)_4$ CONJUGATES

(57) Abstract

Novel trimeric and tetrameric antibodies are disclosed, including bispecific and trispecific $F(ab)_3$ and $F(ab)_4$ antibodies. A simple and efficient method is described for the production of pure $F(ab'\gamma)_3$ antibodies, in which the individual antibody Fab' fragments are joined via stable thioether linkages. Hybrid molecules were constructed from mouse monoclonal antibodies with specificities for targetting cytotoxic effectors (human peripheral blood T cells) against ^{51}Cr-labelled chicken red blood cells. Fab' fragments from two of the chosen antibodies were first coupled via their hinge-region SH groups using o-phenylenedimaleimide (oPDM), this bispecific fragment was then linked, again via the hinge region using oPDM, to a third Fab' fragment.



TRISPECIFIC $F(ab')_3$ ABC

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Bi-or trispecific $(Fab)_3$ or $(Fab)_4$ conjugates

Antibodies which are bispecific with respect to the antigen they recognize have been used successfully in a number of applications. In immunochemistry they have been used to cross-link cellular antigen and detecting agent such as ferritin or horseradish peroxidase, doing away with the antibody conjugates used in more conventional methods. Similarly they have been used as heterobifunctional protein cross-linkers for the immobilization of enzymes in a number of assays. Perhaps their greatest potential lies in their therapeutic use for the targeting of unwanted cells or pathogens by cytotoxic effector cells or pharmacologic agents such as drugs or toxins.

It has been demonstrated that bispecific $F(ab'\gamma)_2$ antibodies, in which one Fab'γ arm is directed at a lymphoma cell and the other binds to a ribosome-inactivating protein, such as ricin A chain or saporin, can target a toxic agent to tumour cells both <u>in vitro</u> and <u>in vivo</u> and prevent further growth. For targeting effector T cells and polymorphonuclear leukocytes, bispecific antibodies have usually have been employed which cross-link the T cell receptor complex or the Fc receptor, respectively, onto the target cell and thereby mediate high levels of specific lysis. By using the appropriate derivatives in this way, it has been possible to show that both normal circulating T cells and single clones of cytotoxic T lymphocytes can be "armed" to destroy almost any specified target cell, and that lysis is independent of the major histocompatibility complex status of the cells involved. Furthermore, the bispecific antibodies do not simply serve to "glue" the two cell populations together, but in linking the effector and target cell actually trigger the lytic process.

In addition to therapeutic uses, bispecific antibodies have also been useful as tools for understanding some of the molecular

interactions which occur when resting T cells are activated for cytotoxicity and proliferation. At this point the relationship between activation of effectors for lysis and the activation for proliferation is not clear. As a general rule effectors, such as T cells, are not cytotoxic when resting but become cytotoxic when profilterating. However, certain effector populations can be activated to become cytotoxic without being driven into proliferation. While monoclonal antibodies directed at the antigen receptor complex (TCR-CD3) on T cells can, to a limited extent at least, mimic antigen in triggering activation for cytotoxicity and proliferation, it has been shown that bispecific antibodies with dual specificity for the TCR-CD3 and one of a group of accessory T cell molecules, such as CD2 CD4 or CD8, are more potent in this respect.

According to a first aspect of the present invention there is provided a trimeric or tetrameric antibody, preferably bispecific or trispecific. By antibody is meant a moiety capable of binding to one or more specific sites on one or more specific antigens. Trimeric antibodies consist of three structurally similar arms, such as three Fab arms, linked together. Tetrameric antibodies consist of four such arms.

Bispecific antibodies of the invention consist of two arms having a first antigen specificity, the third, and fourth if present, having a second antigen specificity. Trispecific antibodies of the invention consists of three arms having, respectively, first, second and third antigen specificities, if present, the fourth arm has the same antigen specificity as one of the first three arms. Preferred antibodies of the invention are $F(ab)_3$ or $F(ab)_4$ antibodies, such as a bispecific or trispecific $F(ab')_3$ or $F(ab')_4$ antibody and particularly preferably a bispecific or trispecific $F(ab'\gamma)_3$ or $F(ab'\gamma)_4$ antibody. By Fab' antibody is meant an Fab antibody fragment which has been generated by pepsin cleavage of an antibody.

Antibody specificity in antibodies according to the invention may, however, be provided by antibody fragments from any source,

including genetically engineered Fv fragments, which may have engineered on multiple residues suitable for forming links between fragments. Such fragments will form Fv_3 and Fv_4 antibodies.

Antibodies of the invention may have specificities for any antigens against which antibodies can be raised or engineered. They find particular application in therapy, especially against tumour cells, but also have applications in assay techniques.

Preferably, however, at least one arm of the antibody specific for a marker on a target, which may be a target cell such as tumour cell, and at least one arm is specific for a marker on an effector, which may be an effector cell such as a T cell, lymphocyte or macrophage, or it may be another cell toxin such as a ribosome-inactivating protein, for example saporin, ricin A chain or intact ricin, or another therapeutic agent to which antibodies can be raised or engineered, such as daunomycin or adriamycin.

In the case of a trispecific antibody, the trispecificity allows it to at once bind to an effector cell and to activate it. The third arm binds to the target cell. It is preferred that two arms of the trispecific antibody are specific to T cells, one of the CD3 molecule and the other to an accessory surface molecule such as the CD2, CD4 or CD8. Alternatively, both arms may be specific for CD2. In that event, the two arms are specific for different epitopes on CD2 such as $T11_2$ and $T11_3$ or $T11_2$ and $T11_3$.

Also in accordance with the first aspect of the invention there is provided a process for the preparation of a bispecific $F(ab)_3$ antibody comprising:

(i) dissociating a first $F(ab)_2$ antibody fragment having a first specificity into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms;

(iii) linking the first Fab arm from step (i) to the second Fab arm from step (ii), the molar ratio of first Fab arm: a second Fab arm being 2:1, to construct a bispecific $F(ab)_3$ antibody.

Also in accordance with the invention there is provided a process for the preparation of a trispecific $F(ab)_3$ antibody as described above comprising:

(i) dissociating a first $F(ab)_2$ antibody fragment having a first specificity into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms;

(iii) linking the first Fab arm from step (i) to the Fab arm from step (ii) to construct a bispecific $F(ab)_2$ antibody;

(iv) dissociating a third $F(ab)_2$ antibody fragment having a third specificity into its two component Fab arms; and

(v) linking the bispecific $F(ab)_2$ antibody from step (iii) to the Fab arm from step (iv) to give specific $F(ab)_3$.

Preferably, the Fab fragments are generated by treating the antibodies providing the fragments with a proteolytic enzyme such as pepsin to give a monospecific $F(ab)_2$ fragment. This is split by reaction with, for example, 2-mercaptoethanol to give Fab_{SH} fragments, in which the -S-S- links between the Fd chains of the $F(ab)_2$ fragments in the original antibody have been broken and reduced to -SH groups. In the preparation Fab specific antibody, Fab_{SH} fragments are prepared from two antibodies to give $FabA_{SH}$ and $FabB_{SH}$ fragments. The linkage of the Fab_{SH} fragments is effected by treating $FabA_{SH}$ fragments with o-phenylenediamine (oPDM) to give $FabA_{mal}$ fragments. These are combined with untreated $FabB_{SH}$ fragments, in a 2:1 weight ratio, under cross-linking conditions to give bispecific FabAAB. The product was, reduced and alkylated with 20mM 2-mercaptoethanol and 25mM iodoacetamide respectively to remove

any minor products which may have formed by oxidation or disulphide exchange, before fractionating an Ultrogel AcA44. In the preparation of trispecific antibody Fab_{SH} fragments are prepared from three antibodies to give $FabA_{SH}$ $FabB_{SH}$ and $FabC_{SH}$ fragments. The linkage of the Fab_{SH} fragments is effected by treating $FabA_{SH}$ fragments with oPDM to give $FabA_{mal}$ fragments. These are combined with untreated $FabB_{SH}$ fragments under cross-linking conditions to give bispecific $F(ab)_2AB$, as shown in Fig. 1. $FabC_{SH}$ fragments are similarly treated with oPDM, to yield $FabC_{mal}$ fragments, which are combined with the $F(ab)_2AB$ by means of an -SH group on the $F(ab)_2AB$ to give trispecific $F(ab)_3ABC$ antibody (see Fig. 2).

According to a second aspect of the invention there is provided a conjugate comprising an antibody according to the first aspect of the invention and an effector for which at least one of the arms of the antibody is specific. The invention also contemplates a process for the preparation of such a conjugate, in which the antibody is mixed with the effector.

The invention further contemplates a pack comprising an antibody according to the first aspect of the invention and, separately, an effector for which at least one of the arms of the antibody is specific.

The invention will be further described with reference to the example and to the figures, in which:

Figure 1 shows the postulated reaction between two Fab fragments to produce a bispecific $F(ab)_2$ antibody;

Figure 2 shows the proposed structure of a trispecific antibody according to the invention;

Figure 3 shows typical chromatography profiles obtained during the preparation of bispecific $F(ab'\gamma)_3$ and trispecific $F(ab'\gamma)_3$;

Figures 4 (a) and (b) are graphs showing redirected cellular cytotoxicity of ^{51}Cr-labelled chicken red blood cells (CRBC) mediated by normal peripheral blood lymphocyte (PBL) and bispecific $F(ab'\gamma)_2$ antibody;

Figure 5 is a graph showing redirected cellular cytotoxicity of ^{51}Cr-labelled CRBC by PBL and one or two bispecific $F(ab'\gamma)_2$ antibodies;

Figure 6 is a graph showing redirected cellular cytotoxicity of ^{51}Cr-labelled CRBC by bispecific $F(ab'\gamma)_2$ and $F(ab'\gamma)_3$ derivatives;

Figure 7 shows the blocking of redirected cellular cytotoxicity mediated by bispecific $F(ab'\gamma)_2$ and $F(ab'\gamma)_3$ derivatives.

Figure 8 is a graph showing redirected cellular cytotoxicity of ^{51}Cr-labelled CRBC by trispecific $F(ab'\gamma)_3$.

Figures 9(a) and (b) are graphs showing the case of blocking of trispecific antibodies;

Figure 10 is a graph showing proliferation (activation) responses of PBL to different Fab'γ derivatives;

Figure 11 shows proliferation responses of PBL to different Fab'γ derivatives in the presence and absence of target cells;

Figure 12 shows the redirected cellular cytotoxocity of human tumour cells (Namalwa) with trispecific antibody; and

Figure 13 shows the redirected cellular cytotoxocity of human tumour cells with a trispecific antibody triggering through CD2.

EXAMPLES

Materials:

All cell culture was performed in supplemented DMEM [Dulbecco's Minimum Essential Medium containing glutamine (200 mM), pyruvate (100 mM), penicillin and streptomycin (100 IU/ml), fungizone (2 μg/ml) and 10% FCS (myoclone) (Giboco Ltd, Paisley, Scotland)], or

in supplemented RPMI [RPMI 1640 Medium containing the same supplements as the DMEM, but with the FCS replaced by 10% normal human serum which had been incubated at 56°C for 30 min to destroy any complement-mediated cytotoxic activity].

Antibodies:

A mouse IgG1 monoclonal antibody, $E_{11}C_{12}$, reacting with chicken red blood cells (CRBC) was raised using conventional hybridoma technology. BALB/c mice were immunized in a protocol which delivered CRBC (approx 10^9) s.c. in CFA and IFA (Difco, Detroit, MI) on days 0 and 14 respectively, and i.p. in DMEM on day 24. Four days later splenic mononuclear cells were fused with the NS-1 (P3/NS-1/1-Ag4.1) mouse myeloma line at a ratio of 2:1 by using a standard somatic fusion protocol with polyethylene glycol 4000 (E.Merck, Darmstadt, Germany). Hybridoma cells secreting anti-CRBC antibody were identified by immunofluorescence staining and flow cytometry as described previously and cloned by limiting dilution.

Additional hybridoma cell lines producing the antibodies OKT1 (CD5), OKT3 (CD3) and OKT11 (CD2) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland), and the hybridoma 3G8 (CD16) was a gift from Dr. D. Segal, NIH, Bethesda, Maryland.

All hybridoma cells were expanded as ascitic tumors in pristane-primed (BALB/c x CBA) F1 mice. The 7S IgG fraction of monoclonal ascites was isolated as described by precipitation in 2 M ammonium sulfate, followed by ion exchange chromatography on Trisacryl-M-DEAE (LKB-Produkter AB, Bromma, Sweden).

Antibody $F(ab'\gamma)_2$ fragments from IgG were prepared by limited proteolysis with pepsin at pH 4.1-4.2 in 0.1 M sodium acetate. The reaction being monitored at regular intervals by rapid fractionation of 100 µg samples on a GF 250 HPLC column (Zorbac), and then, when less than 10% of the IgG remained, the digestion was stopped by adjusting the pH to 8.0 with saturated Tris-base and the products fractionated on Ultrogel AcA44 (LKB).

Example 1: Preparation of bispecific F(ab'γ)$_3$ antibodies:

F(ab'γ)$_2$ from the two chosen mouse antibodies at 10 mg/ml in 0.2 M Tris-HCI buffer, pH 8.0, containing 10 mM EDTA was reduced by addition of 20 mM 2-mercaptoethanol for 30 min at 30°C. At this point both reduced Fab'γ (Fab'γ_{SH}) samples were chilled to 4°C, a temperature which was maintained throughout the remainder of the preparation including the chromatography stages, before running through Sephadex G-25 equilibrated in a buffer of 50 mM sodium acetate, pH 5.3, containing 0.5 mM EDTA. A half volume of 12 mM o-phenylenedimaleimide (oPDM) dissolved in chilled dimethylformamide was then added to one of the two Fab'γ_{SH} samples. After 30 min the maleimidated Fab'γ (Fab'γ_{mal}) was separated from other solutes in the reaction mixture by passage through Sephadex G-25 equilibrated in the 50 mM sodium acetate, pH 5.3, buffer containing 0.5 mM EDTA. It was then added immediately to the Fab'γ_{SH} antibody component of the heterotrimer in a 2:1 molar ratio and concentrated to approximately 5 mg/ml by ultrafiltration under nitrogen using a Diaflo membrane in a chilled Amicon chamber. After incubation for 18 hours, the pH of the reaction mixture was adjusted to 8 using 1 M Tris-HCl, pH 8.0., before reducing with 2-mercaptoethanol at a final concentration of 20 mM for 30 min at 30°C. Finally the bispecific F(ab'γ)$_3$ was separated from other products and residual reactants by passage through Ultrogel AcA44 equilibrated in 0.2 M Tris-HCl. 10 mM EDTA, pH 8.0.

Example 2: Prepartion of trispecific F(ab'γ)$_3$ antibodies:

Firstly, bispecific F(ab'γ)$_2$ antibodies were made by a similar method to that of Example 1, except that the Fab' fragments of Example 1 were mixed in a 1:1 molar ratio, to give bispecific F(ab'γ)$_2$.

Bispecific F(ab'γ)$_2$ was conjugated with a Fab'γ_{mal} from a third antibody. This latter reaction relies on having free -SH groups available in the bispecific F(ab'γ)$_2$ derivative. The procedure and

reaction conditions for linking the Fab'γ_{mal} to the F(ab'γ)$_{2SH}$ were similar to those used Example 1: bispecific F(ab'γ)$_2$ and Fab'γ_2 fragments from the third antibody at 5 mg/ml in 0.2 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA were reduced by addition of 20 mM 2-mercapoethanol for 30 min at 30°C. The samples were chilled to 4°C and run through Sephadex G-25 which had been equilibrated in a buffer of 50 mM sodium acetate, pH 5.3, containing 0.5 mM EDTA. The third Fab'γ_{SH} antibody species was then maleimidated using o-PDM as in the bispecific antibody preparation and finally the bispecific F(ab'γ)$_{2SH}$ and Fab'γ_{mal} were mixed together at a weight ratio of 1:4 for 18 hours at 4°C. Following chormatography on AcA44, 150 kDa-sized material (i.e. F(ab'γ)$_3$) was harvested and concentrated.

Trispecific F(ab'γ)$_4$ was also generated during the trispecific F(ab'γ)$_3$ preparation. It emerged from the AcA44 column at a position which corresponded to that of a protein with a molecular weight of approximately 200 kDa. This size is consistent with the joining of four Fab'γ fragments during the reaction. Apparently, the bispecific F(ab'γ)$_2$ has conjugated with two Fab'γ_{mal} fragments from the third antibody.

The final products in the reaction mixtures were reduced and alkylated with 20 mM 2-mercaptoethanol and 25 mM iodoacetamide respectively to remove any minor products which may have formed by oxidation or disulphide exchange, before fractionating on Ultrogel AcA44.

By these methods, the following antibodies can be prepared:

Bispecific $F(ab)_3$ antibodies

For targeting cytotoxic T cells to unwanted cells:

CD2 x CD2 x target*

CD3 x CD3 x target

CD5 x CD5 x target

CD3 x target x target

Trispecific $F(ab)_3$ antibodies

For targeting cytotoxic T cells to unwanted cells:

CD2 x CD3 x target

CD2 x CD5 x target

CD3 x CD5 x target

CD3 x LFA3 x target

CD3 x CD4 x target

CD3 x CD8 x target

CD2($T11_1$) x CD2'($T11_3$) x target

* target = CRBC or a tumor specific antigen

For targeting cytotoxic agents to unwanted cells:

toxin ** x Ig x CD19

toxin x Ig x CD22

toxin x Ig x CD37

** toxin = ribosome inactivating proteins such as saporin or ricin

The proposed structure of a trispecific F(ab'γ)$_3$ antibody of the invention specific for antigens A, B and C is shown in Fig. 2. Although not shown, some of the γ-L chain disulfide bonds will be reduced during the preparation. Such reduction is known not to compromise antigen binding activity in Fab fragments. Before the final product was alkylated with iodoacetamide, one hinge-region sulphydryl (-SH) group remained, offering the potential for linking at least one more Fab'γ_{mal} fragment yielding F(ab'γ)$_4$, as mentioned above. The groups joined to the cysteinyl sulfur are: Q is carboxyamidomethyl 2, a blocking group; and R is o-phenylenedisuccimimidyl.

Typical chromatography profiles obtained during the preparation of (a) bispecific F(ab'γ)$_3$ and (b) trispecific F(ab'γ)$_3$ derivatives are shown in Fig. 3 (a) and (b) respectively

(a) A reaction mixture containing Fab' $_{mal}$ and Fab' $_{SH}$ at a weight ratio of 2:1 was reduced and alkylated and then fractionated on Ultrogel AcA44 in 0.2 M Tris HCl, pH 8.0. The unreacted Fab' fragments and the bispecific F(ab'γ)$_2$ and F(ab'γ)$_3$ are indicated.

(b) A reaction mixture containing bispecific F(ab'γ) $_{2\ SH}$ and Fab' $_{mal}$ at a weight ratio of 1:4 was reduced and alkylated and then fractionated as for (a). Fab'γ, F(ab'γ)$_2$, F(ab'γ)$_3$ and putative F(ab'γ)$_4$ are indicated.

Target cells:

Chicken red blood cells (CRBC) and Namalwa lymphoma cells were used as targets throughout the study. As regards the CRBC, fresh blood was collected from Rhode Island Red Chickens into preservative-free heparin and washed in DMEM before storing in supplemented DMEM at 4°C. For radiolabelling, 50μl of CRBC ($5x10^8$) or 200μl of Namalwa (2×10^7) were first incubated in 250 μl $Na_2{}^{51}CrO_4$ (Amersham International UK) for 40 min at 37°C and then washed four times in DMEM before resuspending at $2x10^5$/ml in supplemented DMEM.

Effector Cells:

Donors for PBL were healthy laboratory personnel in the age range of 21 to 55 years. Blood was collected into preservative-free heparin and separated by flotation on Ficoll-Hypaque (Lymphoprep, Nyeguard, Oslo, Norway). Cells collected at the interface were washed in phosphate-buffered saline (PBS) and resuspended in supplemented DMEM for use in cytotoxicity or proliferation assays.

Redirected cellular cytotoxicity (RCC) assay:

Cytotoxicity was measured by a standard ^{51}Cr-release assay in 96-well, U-bottomed, microculture plates (Gibco). Each well received 50 μl of antibody diluted in supplemented DMEM, followed by 10^4 ^{51}Cr-labeled CRBC (50 μl) or 10^4 ^{51}Cr-labeled Namalwa cells (50 μl) and $2x10^5$ or $5x10^5$ PBL effectors (100 μl) respectively in supplemented DMEM. The cell mixtures were then sedimented by centrifugation (230xG for five min) before incubating at 37°C in a humidified atmosphere of 5% CO_2 in air for 4, 8 or 21 hours to allow lysis. Finally the cells were sedimented at 420xG for five min and 100-μl aliquots of supernatant removed to assess the release of ^{51}Cr from target cells. Percentages of specific ^{51}Cr release were calculated by the usual method using detergent lysis with 1% Nonidet P40 to give maximum ^{51}Cr release.

Graphs showing redirected antibody dependent cellular cytotoxicity of ^{51}Cr-labeled CRBC mediated by normal PBL and bispecific $F(ab'\delta)_2$ antibody are shown in Fig. 4:

(a) shows lytic activity of the CD3 x CRBC antibody (100 ng/ml) in 4 and 8 hour assays using fresh PBL from six healthy donors.

(b) shows lysis of CRBC using PBL from one healthy donor and various concentrations (as indicated) of CD3 x CRBC and CD16 x CRBC in 4, 8 or 21 hour assays. The assay time is indicated on each of the CD3 x CRBC titration curves, but is omitted from those of the CD16 x CRBC derivative due to their proximity.

Note that while there is considerable variation between donors, all have shown appreciably higher levels of lysis in the longer assay. With two of the donors it was only in this longer 8 hour assay that significant release of ^{51}Cr could be measured at all. This result is confirmed and extended for one donor in Figure 4b, which shows that near maximal lytic activity was approached in eight hours with an antibody concentration of 100 ng/ml.

A graph showing redirected antibody dependent cellular cytotoxicity of ^{51}Cr-labeled CRBC by PBL and one or more bispecific $F(ab'\gamma)_2$ antibodies is shown in Fig. 5. Lysis was measured in an 8 hour assay using fresh PBL from one donor and the derivatives indicated.

Results indicated that while the CD3 x CRBC $F(ab'\gamma)_2$ lysed CRBC at concentrations as low as 4 ng/ml, almost 1000 times more CD2 x CRBC $F(ab')_2$ was necessary to achieve appreciable levels of cytotoxicity. Mixing the CD2 bispecific reagents with the CD3 x CRBC antibody resulted in no additive effects, giving similar levels of killing to those seen with the CD3 derivative alone.

Fig. 6 shows that the redirected cellular cytotoxicity against ^{51}Cr-labelled CRBC is considerably higher for bispecific $F(ab'\gamma)_3$ derivatives than for bispecific $F(ab'\gamma)_2$ derivatives. For example, the CD3 x CD3 x CRBC trimer was found to be up to 125 times more potent than the equivalent dimer, CD3 x CRBC, giving significant activity at concentrations below 0.1ng/ml.

The efficiency of these bispecific trimers is not dependant upon two Fab'δ arms being bound to the effector cells, since the derivative CD3 x CRBC x CRBC gives a similar cytotoxicity result. The increased efficiency probably results from the increased binding avidity of the bispecific trimers. Blocking studies with excess Fab'δ (Fig. 7) showed that the redirected cellular cytotoxicity mediated by a trispecific trimer is far more readily inhibited by blocking with the appropriate Fab'δ antibody than is that of a bispecific trimer.

Fig. 7a shows a comparison of the cytotoxicity of CD2 x CRBC antibody with that of CD2 x CD2 x CRBC antibody, blocked with CD2 antibody. Fig. 7b shows a comparison of the cytotoxicity of CD3 x CRBC antibody with that of CD3 x CD3 x CRBC antibody, blocked with CD3 antibody.

These graphs show that redirected cellular cytotoxicity mediated by bispecific dimers is completely inhibited by the addition of 25μg/ml of the appropriate Fab'δ, whilst under similar conditions bispecific trimers still showed cytotoxic activity with blocking Fab'δ at 500μg/ml.

A graph showing redirected antibody dependent cellular cytotoxicity of ^{51}Cr-labelled CRBC by trispecific F(ab'δ)$_3$ is shown in Fig. 8. Lysis was measured in an 8 hour cytotoxicity assay using fresh PBL from one donor and the derivatives indicated.

The results show that a trispecific F(ab'δ)$_3$ derivative containing anti-CD2 and -CD3 antibody specificities was at least 100 times more potent than the best bispecific F(ab'δ)$_2$ derivative and could promote significant levels of lysis at concentrations well below 0.1 ng/ml.

From these results, it appears that trispecific F(ab)$_3$ antibodies according to the invention when used therapeutically will be effective at doses as low as 1% of those required for similar bispecific F(ab)$_2$ antibodies. This has clear advantages both in

cost of treatment and directly to the patient who will take a lower dosage of antibody.

To confirm that both the arms of the trispecific antibody specific for the effector were required for full activity we performed RCC assays in the presence of blocking Fab' δ antibody. Figure 9(a), shows the cytotoxicity of the CD2 x CD3 x CRBC antibody in the presence of, respectively, CD2 antibody, CD3 antibody and a mixture of CD2 antibody and CD3 antibody as blocker. The graph shows that when using CD2 antibody CD3 antibody at 500 μg/ml, a concentration known to be sufficient to block bispecific derivatives (Fig. 7) no reduction in the activity of this trispecific reagent was observed. It was only when both these blocking antibodies were included in the assay that any reduction in activity occurred. With Fab' from both CD2 antibody and CD3 antibody each 500μg/ml, specific ^{51}Cr release was reduced from 65% to 15%. In a similar experiment the results of which appear in Fig. 9(b), the trispecific $F(ab'\gamma)_3$ antibody CD3 x CD5 x CRBC also demonstrated redirected cellular cytotoxicity which was much more resistant to blocking than a bispecific $F(ab'\gamma)_2$ (See Fig. 7). In this example however, the blocking was slightly more effective than that with the CD2 x CD3 x CRBC reagent. While this increased sensitivity to blocking could reflect a reduced avidity by the CD3 x CD5 x CRBC antibody, it is probably also affected by the relatively poor cytotoxic potency of this reagent.

Incorporation of [^{3}H]thymidine:

Proliferation of normal T cells in response to mitogenic antibody derivatives was assessed in vitro. Peripheral blood lymphocytes (PBL) isolated from Ficoll-Hypaque were cultured at 37^{o}C in 96-well, U-bottomed, microculture plates (10^5/well) in supplemented RPMI containing the various antibody derivatives, together with or without CRBC (200μl /well). After 48 hours each well was pulsed for 16 hours at 37^{o}C with 1μCi [^{3}H]thymidine (Amersham) and the incorporated radioactivity harvested onto glass microfibre filters and assessed as described previously. All experimental points were determined in triplicate.

A graph showing proliferation (activation) responses of PBL to different Fab'γ derivatives is shown in Figs. 10 and 11. Fresh PBL from one donor were cultured for 48 hours with the Fab'γ derivative or mixture of derivatives indicated.

The results show that in the absence of CRBC only the trispecific antibody, CD2 x CD3 x CRBC, and to a lesser extent CD3 x CD5 x CRBC, gave a significant proliferation signal. In contrast when CRBC were included in the cultures a number of antibodies, particularly CD2 x CD2 x CRBC and a mixture of CD2 x CRBC + CD3 x CRBC were highly mitogenic.

Bispecific or trispecific $F(ab)_3$ antibodies with the appropriate specificities can be constructed which will first activate cellular effectors, such as T cell or monocytes, and then target them to destroy any unwanted cell. In the case of trispecific antibodies, efficient activation would appear to benefit from two antibody specificities reacting with molecules on the surface of the effector cell. The third Fab specificity of the trispecific $F(ab)_3$ is then available to target against any unwanted cell. Target cells in this system could include neoplastic cells, virally infected host cells (including HIV), autoreactive host cells (B or T lymphocytes) or invading pathogens, including bacteria and viruses.

The bispecific $F(ab'\gamma)_3$ antibodies approximately 100 times more potent than the equivalent bispecific $F(ab'\gamma)_2$ antibodies. For example, CD3 x CD3 x CRBC antibody was still fully active at below 1ng/ml. This surprising increase in performance does not appear to arise from more efficient triggering of cytotoxic T cells, at least as judged by triggering of mitosis. An $F(ab'\gamma)_3$ antibody containing two CRBC specific Fab'γ arms and one T cell specific Fab'γ arm also demonstrated a similar improvement in performance. It seems likely that bispecific $F(ab'\gamma)_3$ antibodies, because they bind to one cell surface through two Fab'γ arms, couple target and effector cells together with an increased avidity over equivalent bispecific $F(ab'\gamma)_2$ antibody. Blocking studies were consistent with this interpretation, showing that the two arms of a bispecific $F(ab'\gamma)_3$

required at least 20 times more free Fab'δ from the appropriate antibody to inhibit cytotoxicity than did equivalent $F(ab'\delta)_2$ reagent.

In the case of trispecific antibodies, it may be advantageous to have two antibody Fab arms reacting with the target cell and one Fab arm recruiting either a cellular effector or a pharmacological agent such as a toxin. As with the effector T cell, other cells, including B cells and monocytes, can be activated when bound by two antibodies reacting with the appropriate surface molecules. Activiated cells which show a high proliferative rate are also often more susceptible to destruction by pharmacological agents such as cytotoxic drugs and toxins; the more rapidly growing tumours, such as childhood acute lymphoblastic leukaemia, are often the most sensitive to conventional chemotherapy. Thus, a trispecific antibody, with two Fab arms directed at the target cell and one at a pharmacological agent, could first activate the target cell and then deliver a poison while it remained in a hypersensitive state.

In addition to the advantages of being able to activate effector or target cells, trispecific antibodies, because they have two Fab arms binding to one surface, also display an increased avidity for that surface. A trispecific $F(ab)_3$ derivative will cross-link two cell surfaces together significantly more strongly than a mixture of two bispecific $F(ab)_2$ antibodies. This advantage may also be applied to immunoassays including enzyme-linked immunosorbent assays or radio-immunoassays. In this situation a trispecific antibody with two different binding sites for a single antigen, such as an enzyme, protein or peptide, and a third Fab arm for a second protein, can be used as a single step cross-linker increased avidity.

Other immunoassays in which a trispecific $F(ab)_3$ derivatives may be useful include situations where it is necessary to capture three different immunogenic antigens, such as enzymes, proteins or peptides, into a tight immune complex. An enzyme and its substrate could be captured directly from solution onto a solid surface in this way.

Redirected cellular cytotoxicity (RCC) of human tumour cells (Namalwa) with trispecific antibody.

Fresh human blood mononuclear cells (PBL) and ^{51}Cr-labelled Namalwa cells were mixed in a standard 20 h cytotoxicity assay with derivatives of the shown specificities. The differentiation antigen CD37 was used as a tumour marker for delivering effectors to the Namalwa cells. The results are shown in Figure 12 of four RCC assays, using PBL from different donors (donor 1-4). In each case the trispecific antibody [anti-CD2 x anti-CD3 x anti-CD37] is far more active than any bispecific antibody. Note that this enhance lysis is specific in that the derivative [anti-CD2 x anti-CD3 x anti-CRBC], which is mitogenic to T cells and was highly potent in RCC against CRBC (Fig. 8), showed no cytotoxic activity (Donor 2). Also a mixture of two bispecific antibodies, [anti-CD3 x anti-CD37] + [anti-CD2 x anti-CD37], was no more active than [anti-CD3 x anti-CD37] alone (Donor 3). These results confirm the CRBC results by showing that a derivative with a pair of antibody arms (anti-CD2 x anti-CD3) which activate cytotoxic T cells, become highly potent reagents for killing unwanted targets when converted into a trispecific antibody by the addition of an anti-target cell arm.

RCC of human tumour cells with a trispecific antibody triggering through CD2.

The experimental conditions were the same as those used in Figure 12, but using effectors from two different donors and derivatives with the specificities shown in Figure 13. The results in Figure 13 show that, for both donors a trispecific antibody derivative containing a mitogenic pair of anti-CD2 antibodies, anti-CD2 ($T11_1$) and anti-CD2' ($T11_3$), is as, or more, active than the original trispecific antibody [anti-CD2 x anti-CD3 x anti-CD37]. Note that three other trimeric antibodies, [anti-CD3 x anti-CD3 anti-CD37], [anti-CD3 x anti-CD4 x anti-CD37] and [anti-CD3 x anti-CD5 x anti-CD37], which are not mitogenic to T cells do not show the enhance activity of the [CD2 x CD3 x CD37] and [CD2 x CD2' xCD37] derivatives, but are probably more active than a bispecific $F(ab')_2$ [anti-CD3 x anti-CD37].

Claims

1. A trimeric or tetrameric antibody.

2. A bispecific or trispecific antibody according to claim 1.

3. An antibody according to any preceding claim comprising at least one arm specific for a marker on a first moiety and at least one arm specific for a marker on a second moiety.

4. A trispecific antibody according to any preceding claim comprising one arm specific for a marker on a first moiety and two arms specific, respectively, for different markers on a second moiety.

5. An antibody according to claim 3 or 4 in which the first and second moieties are, respectively, a target cell and an effector.

6. An antibody according to claims 3 or 4 in which the first and second moieties are, respectively, an effector and a target cell.

7. A antibody according to claim 5 or 6 in which the effector is an effector cell.

8. An antibody according to claims 5 or 6 in which the effector is a therapeutic agent effective to destroy the target cell.

9. An antibody according to claim 8, in which the therapeutic agent is a conventional chemotherapeutic compound to which antibodies can be raised, such as daunomycin or adriamycin.

10. An antibody according to any of claims 5 to 9 in which the target cell is a tumour cell.

11. A trispecific antibody according to any of claims 1 to 5, in which two arms are specific for, respectively, TCR-CD3 and an accessory molecule on effector T cells.

12. A trispecific antibody according to any of claims 1 to 5 comprising two arms specific for, respectively, different sites on a Type 1 or Type 2 ribosome-inactivating protein and at least one arm specific for a marker on a target cell.

13. A trispecific antibody according to any of claims 1 to 4, 6 and 8 to 10 comprising two arms specific for, respectively, two different markers on a target cell and at least one arm specific for a Type 1 or Type 2 ribosome-inactivating protein.

14. A trispecific antibody according to claim 12 or 13, in which the ribosome-inactivating protein is saporin, ricin A chain or intact ricin.

15. A bispecific antibody according to any of claims 1 to 3, 5 to 7 and 10 comprising two arms specific for TCR-CD3.

16. A bispecific antibody according to any of claims 1 to 3, and 5 comprising two arms specific for a target cell.

17. An antibody according to any preceding claim in which the monomer arms are linked by a -S-(o-phenylenedisuccinimidyl)-S- group.

18. A $F(ab)_3$ or $F(ab)_4$ antibody according to any preceding claim.

19. An $F(ab^{\gamma})_3$ or $F(ab^{\gamma})_4$ antibody according to any preceding claim.

20. An $F(ab')_3$ or $F(ab')_4$ antibody according to any preceding claim.

21. An $F(ab'\delta)_3$ or $F(ab'\delta)_4$ antibody according to any preceding claim.

22. A Fv_3 or Fv_4 antibody according to any of claims 1 to 17.

23. A process for the preparation of a bispecific $F(ab)_3$ antibody comprising:

(i) dissociating an $F(ab)_2$ antibody fragment having a first specificty into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms; and

(iii) linking the first Fab arm from step (i) to two component Fab arms from step (ii) to give bispecific $F(ab)_3$.

24. A process for the preparation of a trispecific $F(ab)_3$ antibody comprising:

(i) dissociating an $F(ab)_2$ antibody fragment having a first specificity into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms;

(iii) linking the first Fab arm from step (i) to the Fab arm from step (ii) to construct a bispecific $F(ab)_2$ antibody;

(iv) dissociating a third $F(ab)_2$ antibody fragment having a third specificity into its two component Fab arms; and

(v) linking the bispecific $F(ab)_2$ antibody from step (iii) to the Fab arm from step (iv) to give trispecific $F(ab)_3$.

25. A process according to claim 23 or 24 in which the $F(ab)_2$ antibody fragments used in steps (i), (ii) and (iv) are obtained by treating antibodies having the said specificities with pepsin or other proteolytic enzyme.

26. A process according to any of claims 23 to 25 in which one or more of steps (i), (ii) and (iv) are carried out by treatment of the $F(ab)_2$ antibody fragment with a reducing agent such as 2-mercaptoethanol.

27. A process according to any of claims 23 to 26 in which the linking of step (iii) is effected by treating the dissociated Fab fragment produced in one of steps (i) and (ii) with o-phenylenedimaleimide and combining the thus treated Fab fragment with the untreated Fab fragment produced in the other of steps (i) and (ii) under cross-linking conditions to give the bispecific $F(ab)_2$ fragment.

28. A process according to any of claims 24 to 27 for the reporting of a trispecific $F(ab)_3$ antibody in which the linking of step (v) is effected by treating the Fab fragment produced in step (iv) with o-phenyleledimaleimide and combining the thus treated Fab fragment with the bispecific $F(ab)_2$ fragment produced in step (iii) under cross linking conditions to give $F(ab)_3$.

29. A conjugate comprising a bispecific or trispecific antibody according to any of claims 1 to 22 or produced by a process according to any of claims 23 to 28 complexed with an effector to which at least one arm of the antibody is specific.

30. A process for the preparation of a conjugate according to claim 29, comprising mixing the antibody with the effector.

31. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as active ingredient, either: a bispecific or trispecific antibody according to any of claims 1 to 22; a bispecific or trispecific antibody produced by a process

according to any of claims 23 to 28; or a conjugate according to claim 29, or a conjugate produced by a process according to claim 30.

32. A pack comprising: a bispecific or trispecific antibody according to any of claim 1 to 22 or produced by a process according to any one of claims 23 to 28 and, separately therefrom, an effector for which at least one arm of the antibody is specific.

Fig. 1.

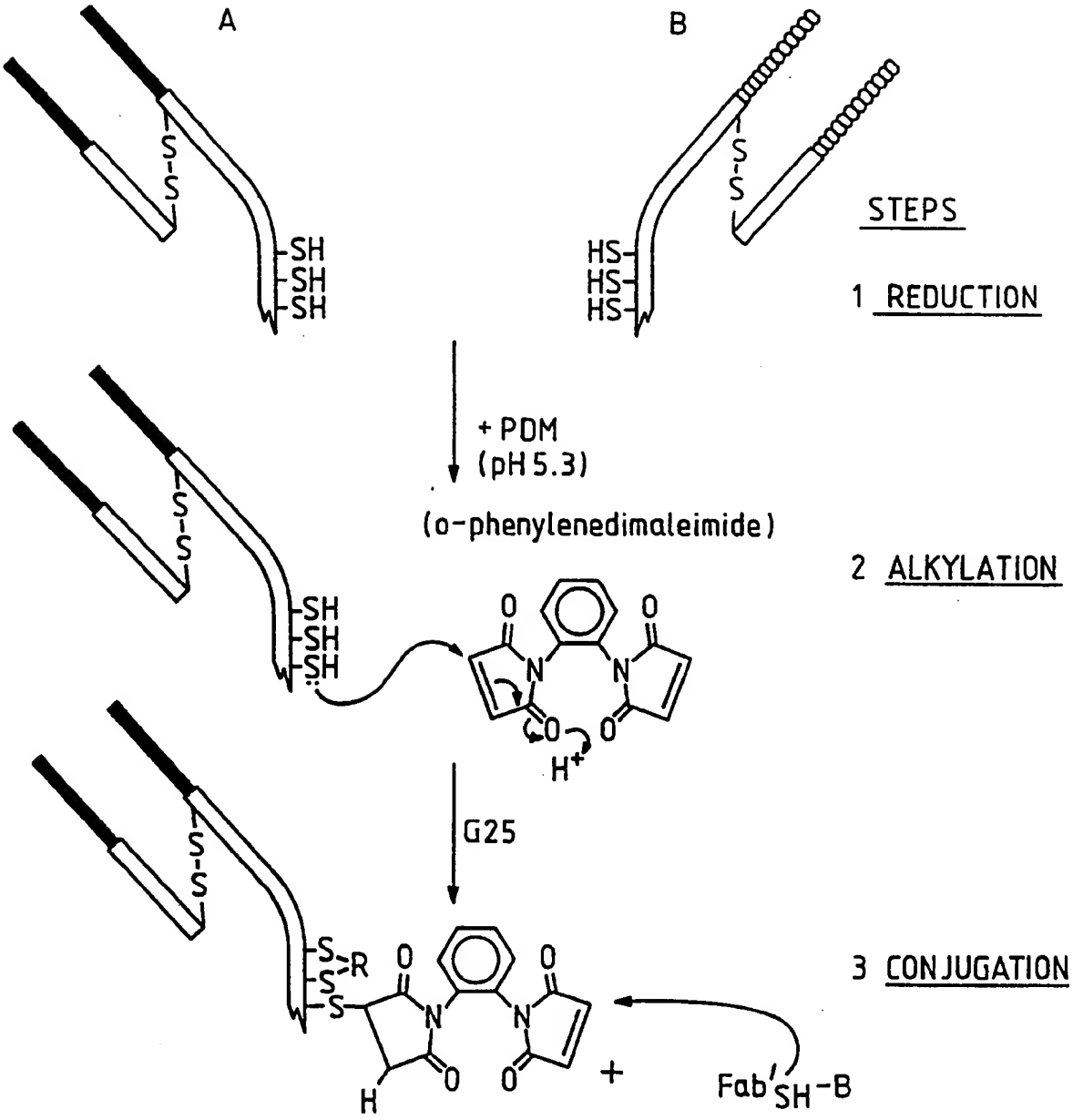
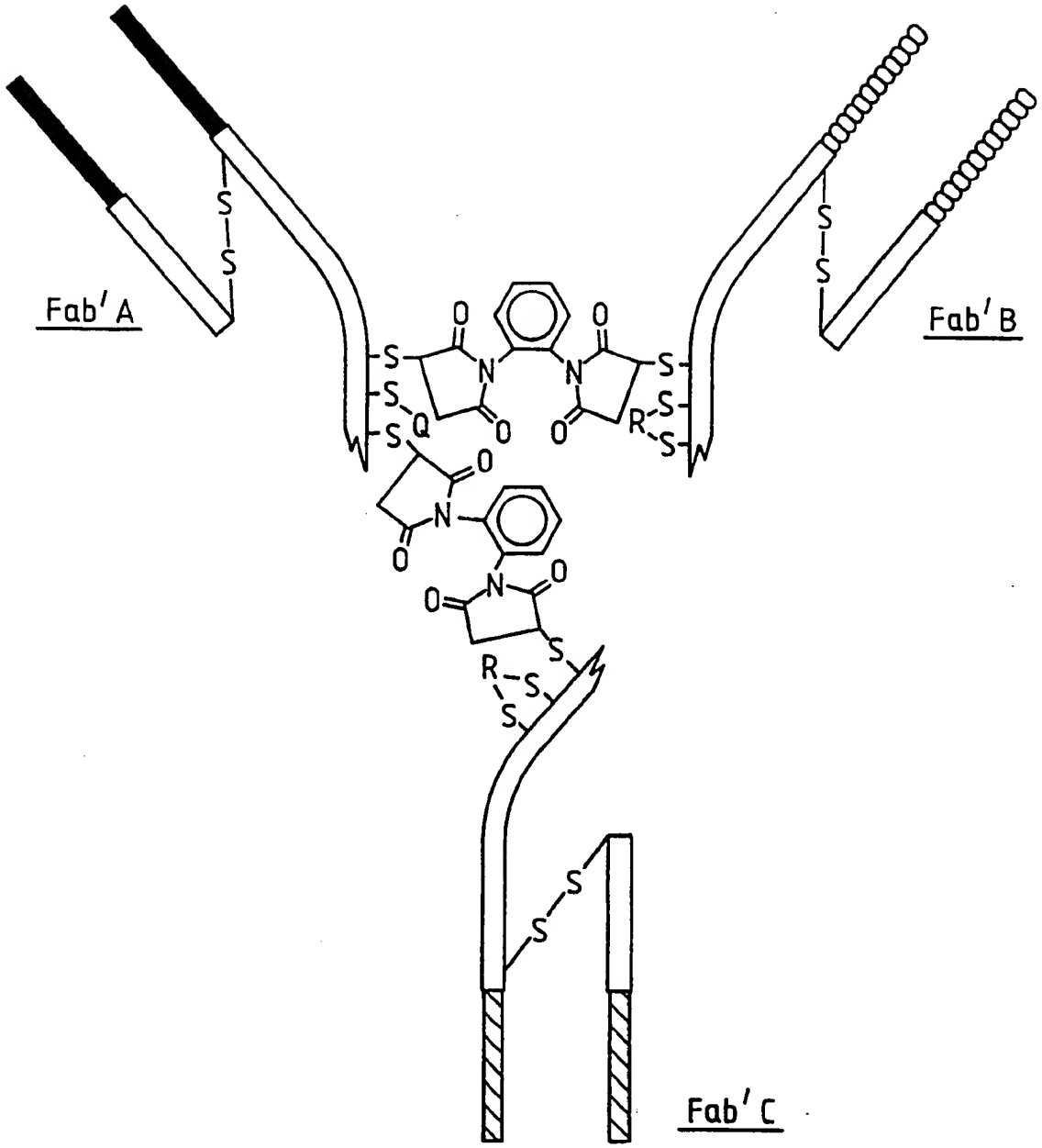

A
B
STEPS
1 REDUCTION
+ PDM
(pH 5.3)
(o-phenylenedimaleimide)
2 ALKYLATION
G25
3 CONJUGATION
Fab'SH-B

Fig. 2.



TRISPECIFIC $F(ab')_3$ ABC

Fig. 3.

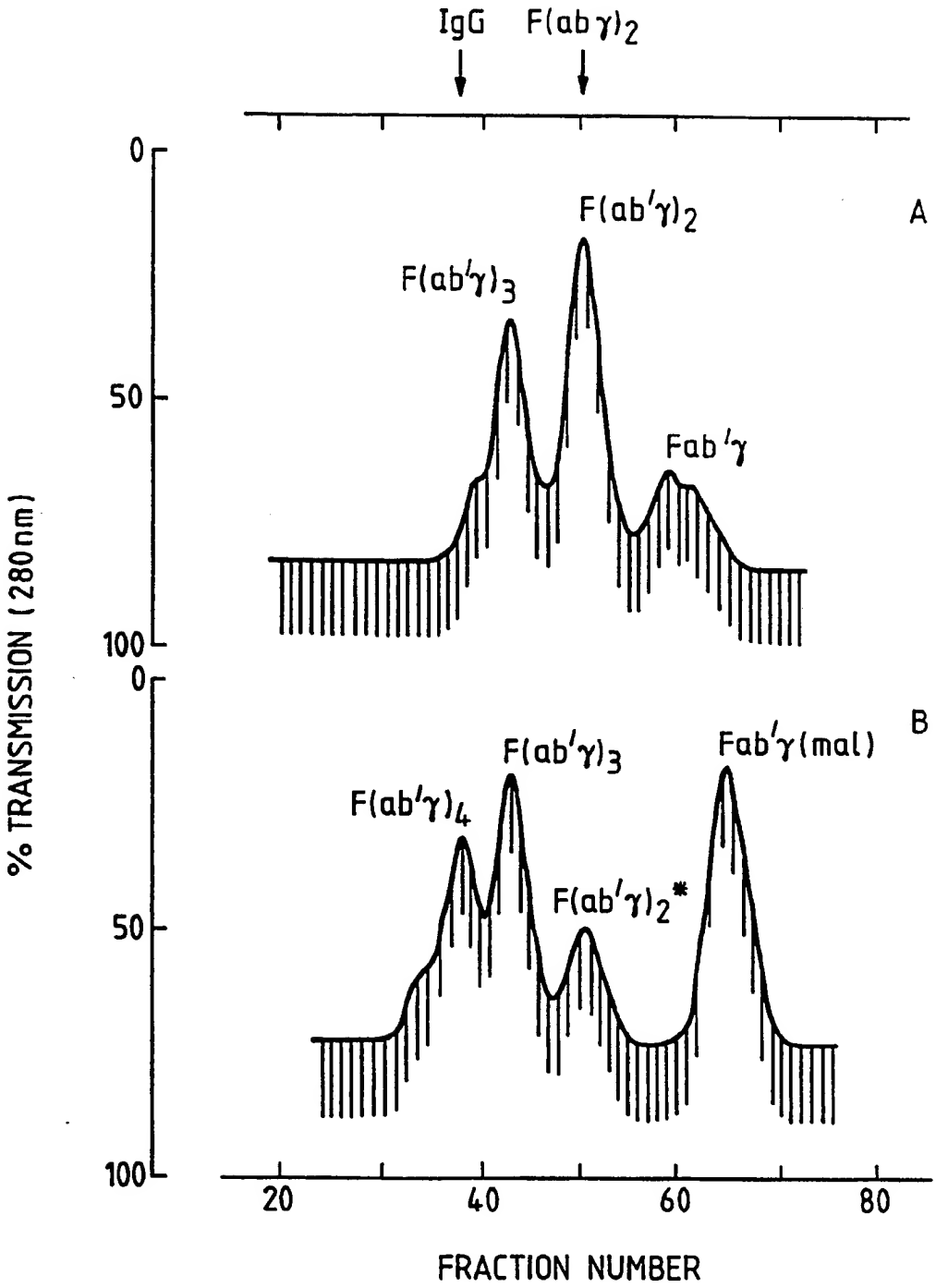

IgG
F(ab γ)2
0
50
100
F(ab'γ)2
A
F(ab'γ)3
Fab'γ
0
50
100
B
F(ab'γ)3
Fab'γ(mal)
F(ab'γ)4
F(ab'γ)2*
% TRANSMISSION (280nm)
20
40
60
80
FRACTION NUMBER

Fig. 4.

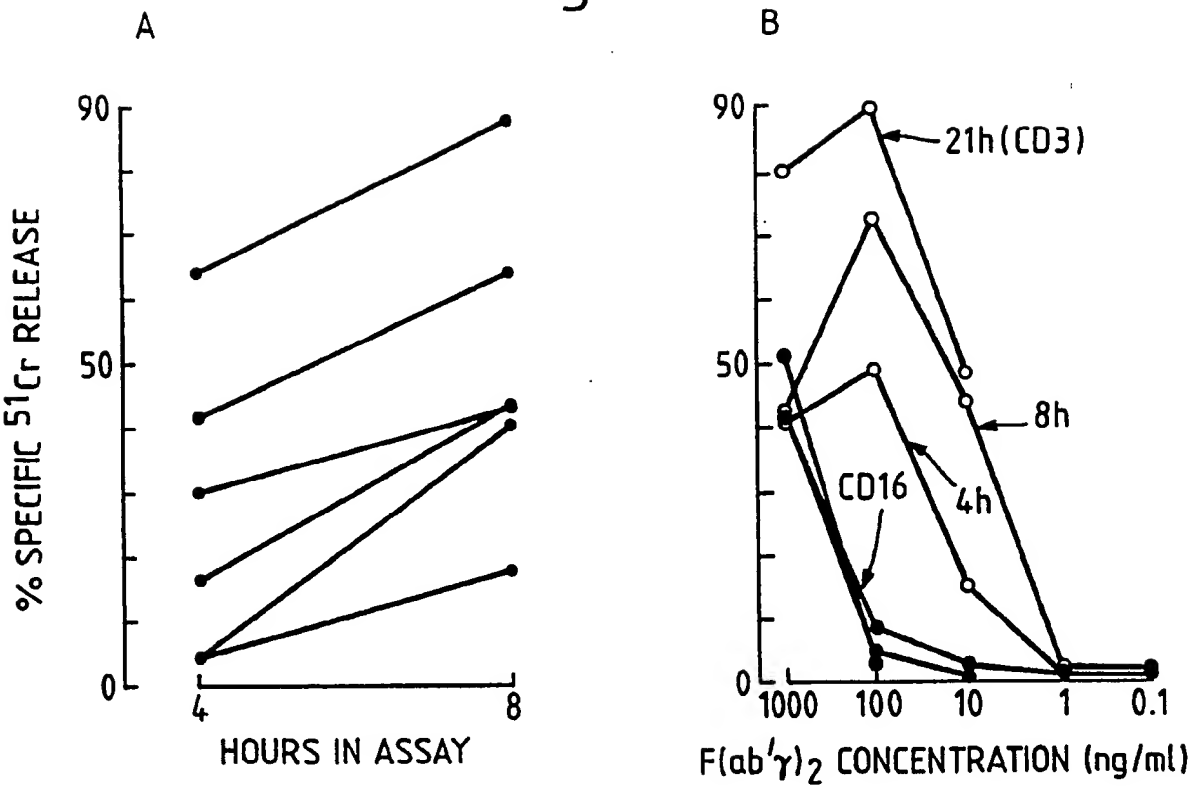

A
90
50
0
% SPECIFIC ^{51}Cr RELEASE
4
8
HOURS IN ASSAY
B
90
50
0
21h (CD3)
8h
4h
CD16
1000
100
10
1
0.1
F(ab'γ)2 CONCENTRATION (ng/ml)

Fig.5.

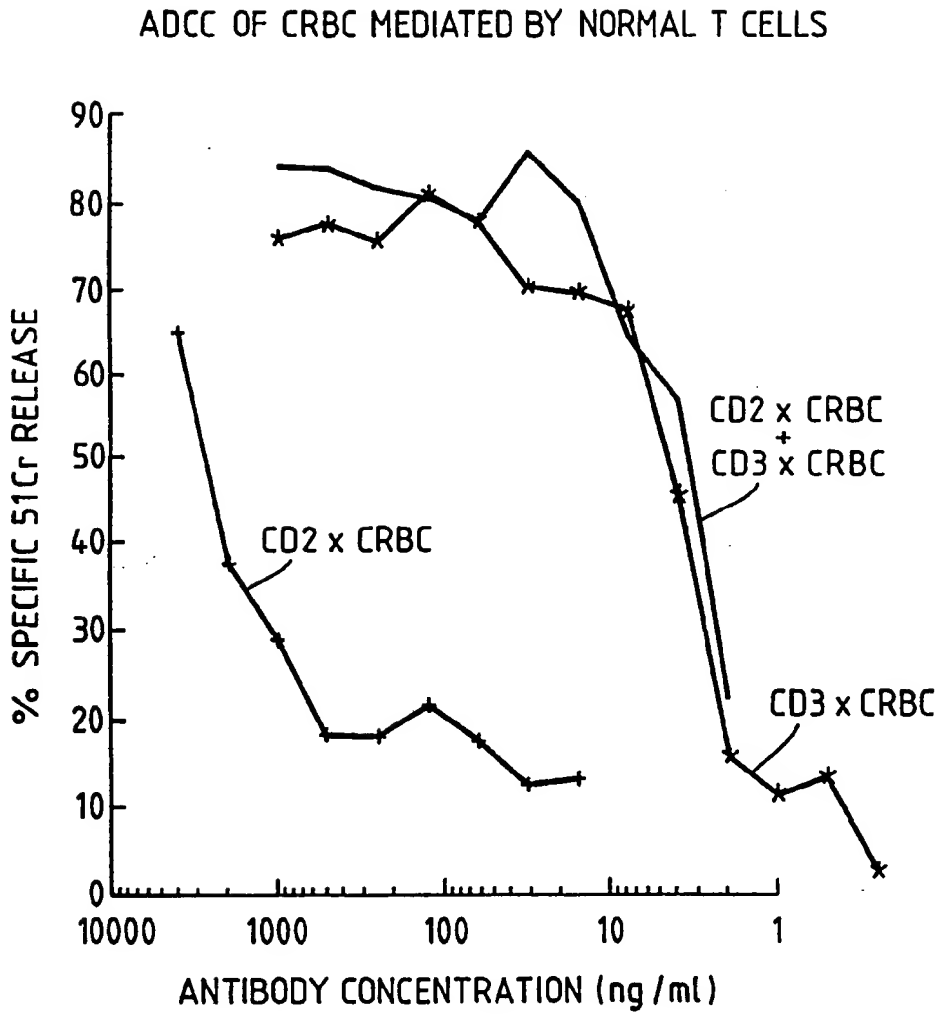

ADCC OF CRBC MEDIATED BY NORMAL T CELLS
% SPECIFIC 51Cr RELEASE
90
80
70
60
50
40
30
20
10
0
10000
1000
100
10
1
ANTIBODY CONCENTRATION (ng/ml)
CD2 x CRBC
+
CD3 x CRBC
CD2 x CRBC
CD3 x CRBC

Fig. 6.

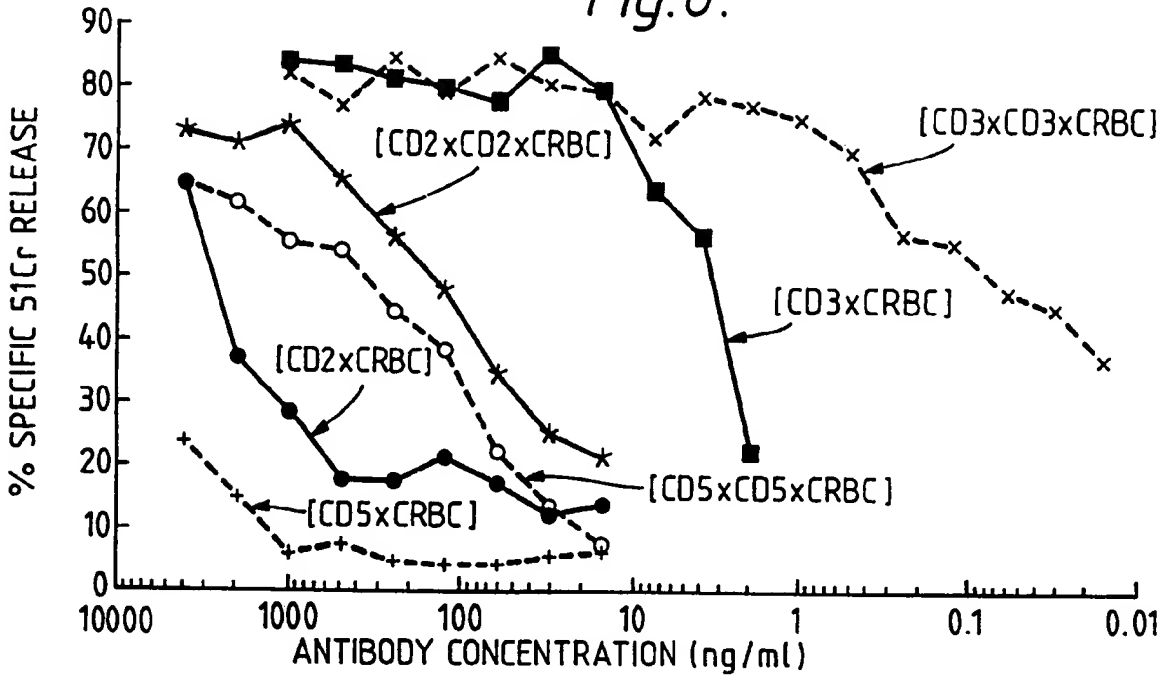

% SPECIFIC 51Cr RELEASE
90
80
70
60
50
40
30
20
10
0
[CD2xCD2xCRBC]
[CD3xCD3xCRBC]
[CD3xCRBC]
[CD2xCRBC]
[CD5xCD5xCRBC]
[CD5xCRBC]
10000
1000
100
10
1
0.1
0.01
ANTIBODY CONCENTRATION (ng/ml)

Fig. 7.

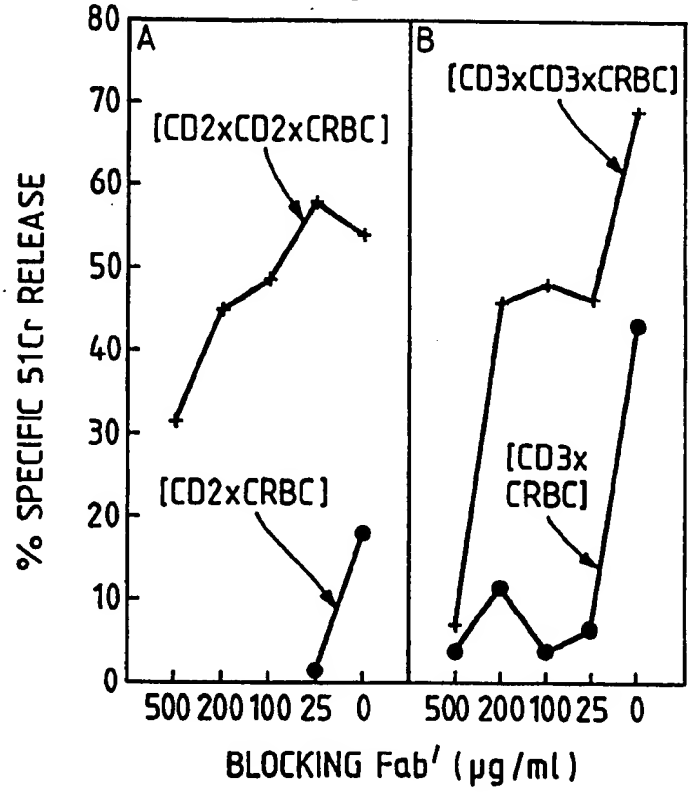

% SPECIFIC 51Cr RELEASE
80
70
60
50
40
30
20
10
0
A
B
[CD2xCD2xCRBC]
[CD3xCD3xCRBC]
[CD2xCRBC]
[CD3x
CRBC]
500 200 100 25 0
500 200 100 25 0
BLOCKING Fab' (µg/ml)

Fig.8.

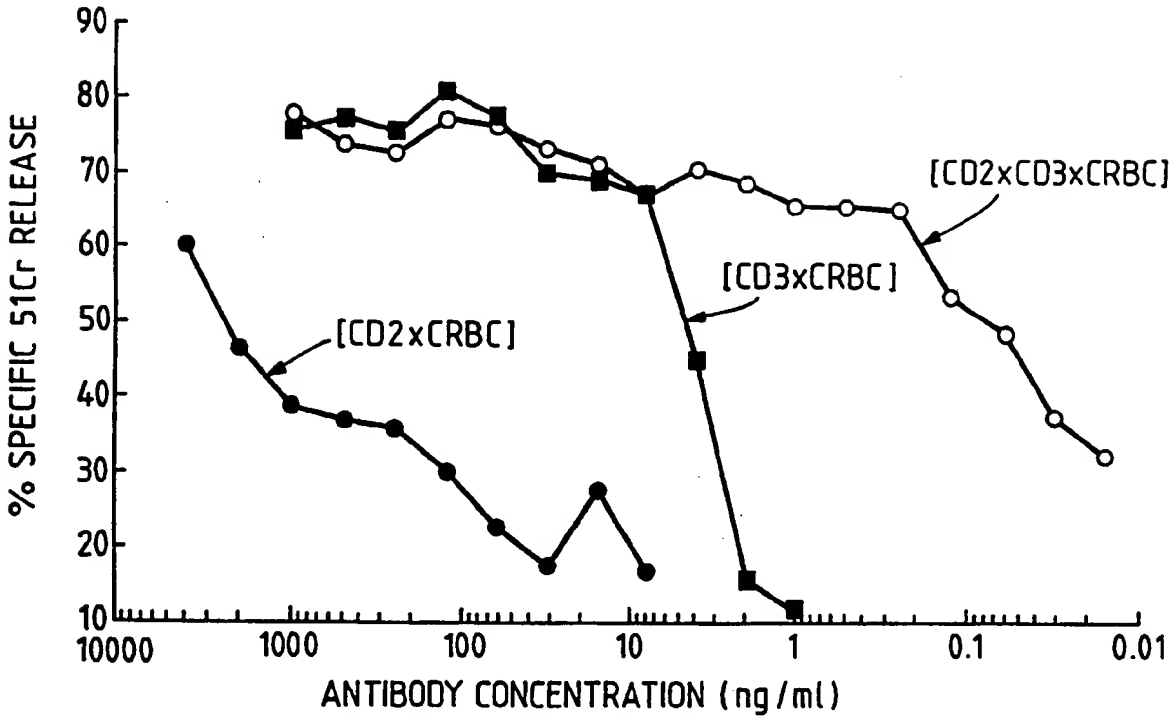

% SPECIFIC 51Cr RELEASE
90
80
70
60
50
40
30
20
10
10000
1000
100
10
1
0.1
0.01
ANTIBODY CONCENTRATION (ng/ml)
[CD2xCD3xCRBC]
[CD3xCRBC]
[CD2xCRBC]

Fig. 9.

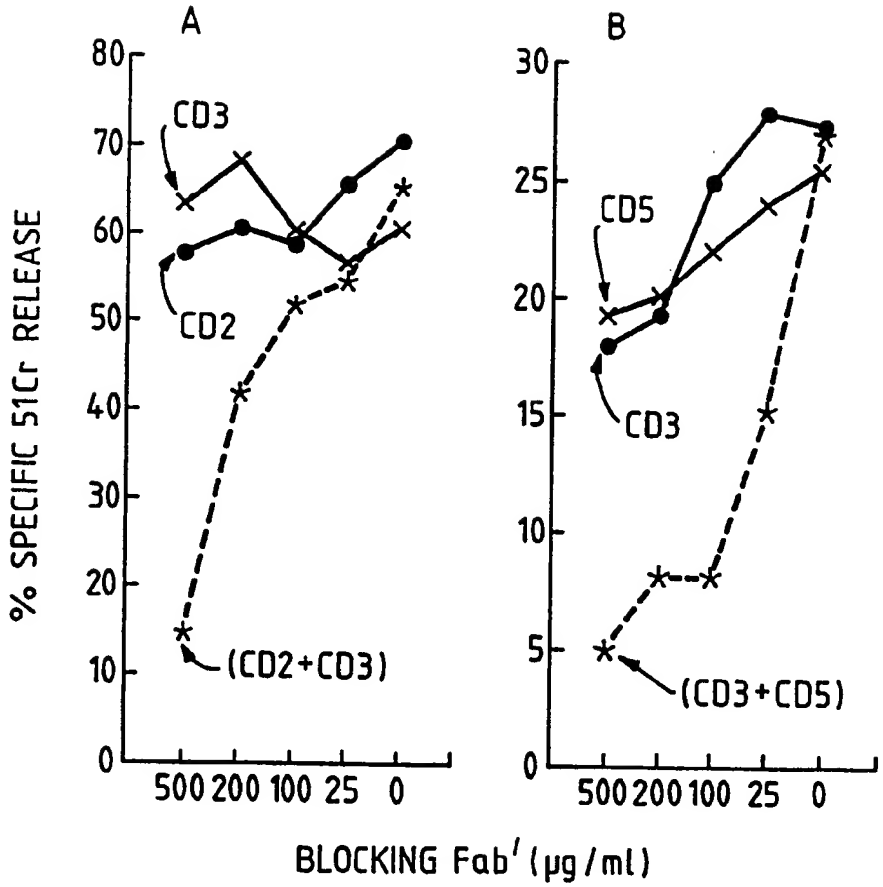
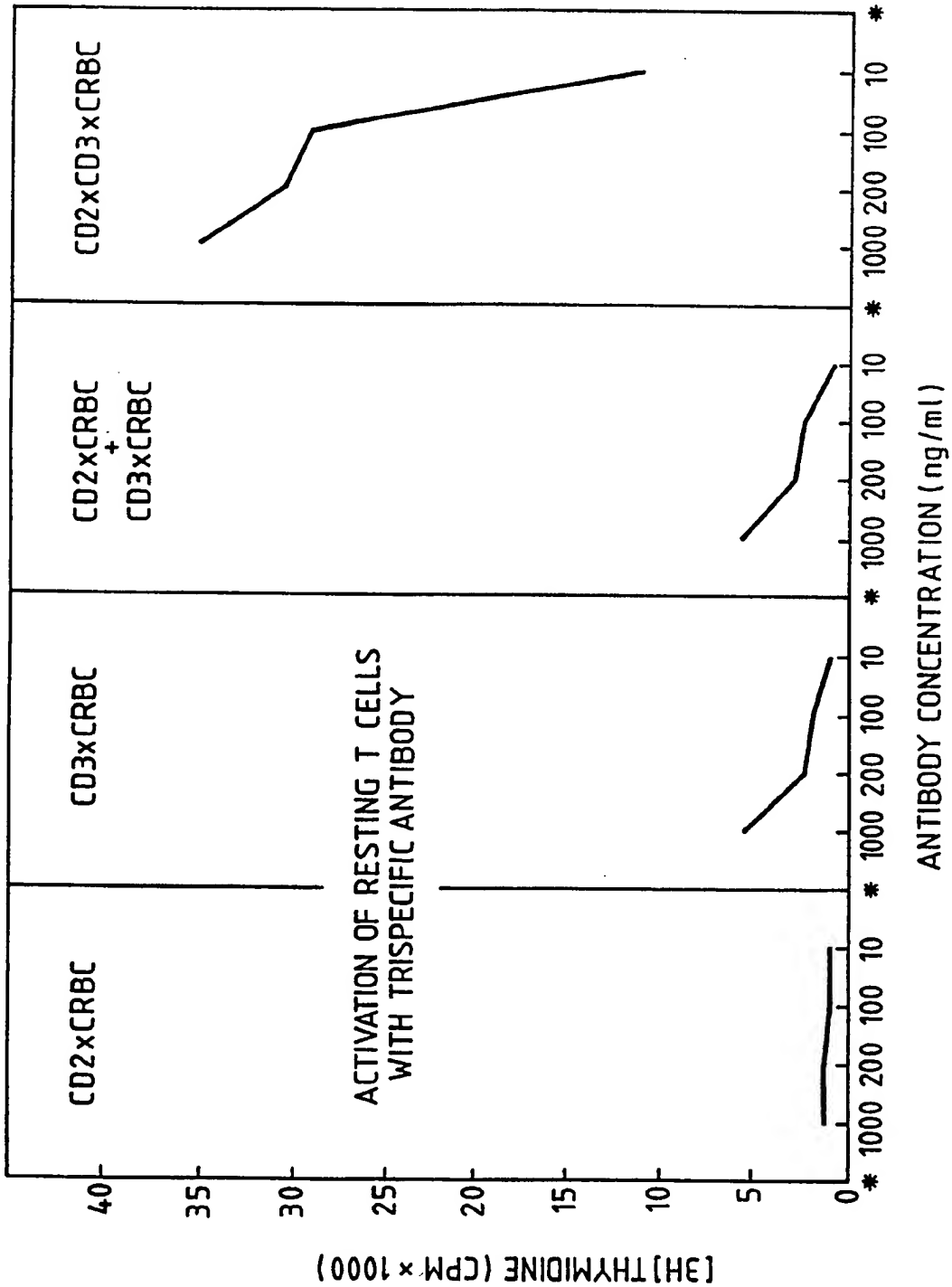

A
B
80
70
60
50
40
30
20
10
0
30
25
20
15
10
5
0
CD3
CD2
(CD2+CD3)
CD5
CD3
(CD3+CD5)
500 200 100 25 0
500 200 100 25 0
% SPECIFIC 51Cr RELEASE
BLOCKING Fab' (µg/ml)

Fig. 10.


ACTIVATION OF RESTING T CELLS WITH TRISPECIFIC ANTIBODY
CD2xCRBC
CD3xCRBC
CD2xCRBC + CD3xCRBC
CD2xCD3xCRBC
[3H] THYMIDINE (CPM x 1000)
0
5
10
15
20
25
30
35
40
* 1000 200 100 10 *
ANTIBODY CONCENTRATION (ng/ml)

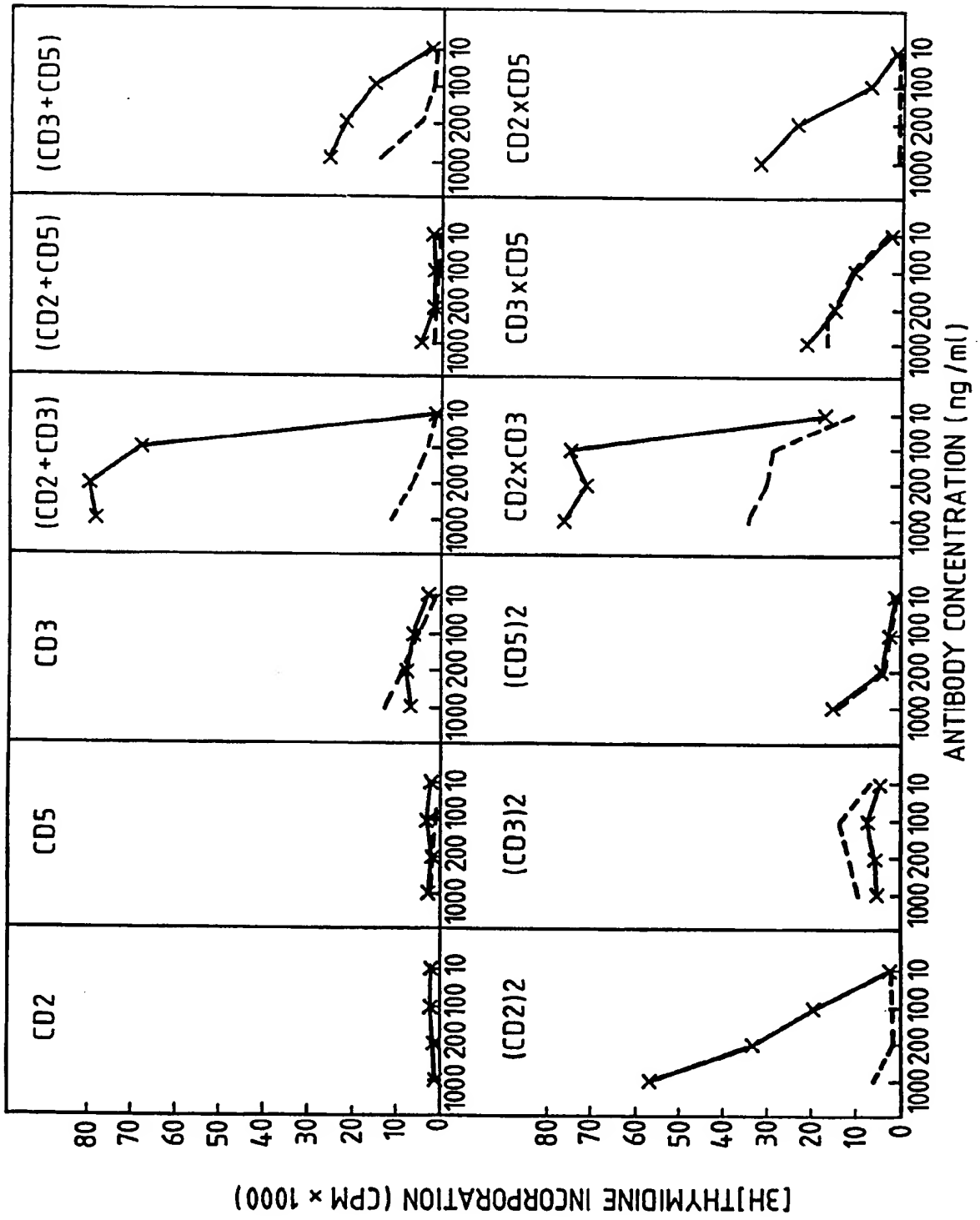

CD2
CD5
CD3
(CD2+CD3)
(CD2+CD5)
(CD3+CD5)
(CD2)2
(CD3)2
(CD5)2
CD2xCD3
CD3xCD5
CD2xCD5
0
10
20
30
40
50
60
70
80
1000 200 100 10
ANTIBODY CONCENTRATION (ng /ml)
[3H]THYMIDINE INCORPORATION (CPM x 1000)

Fig. 11.

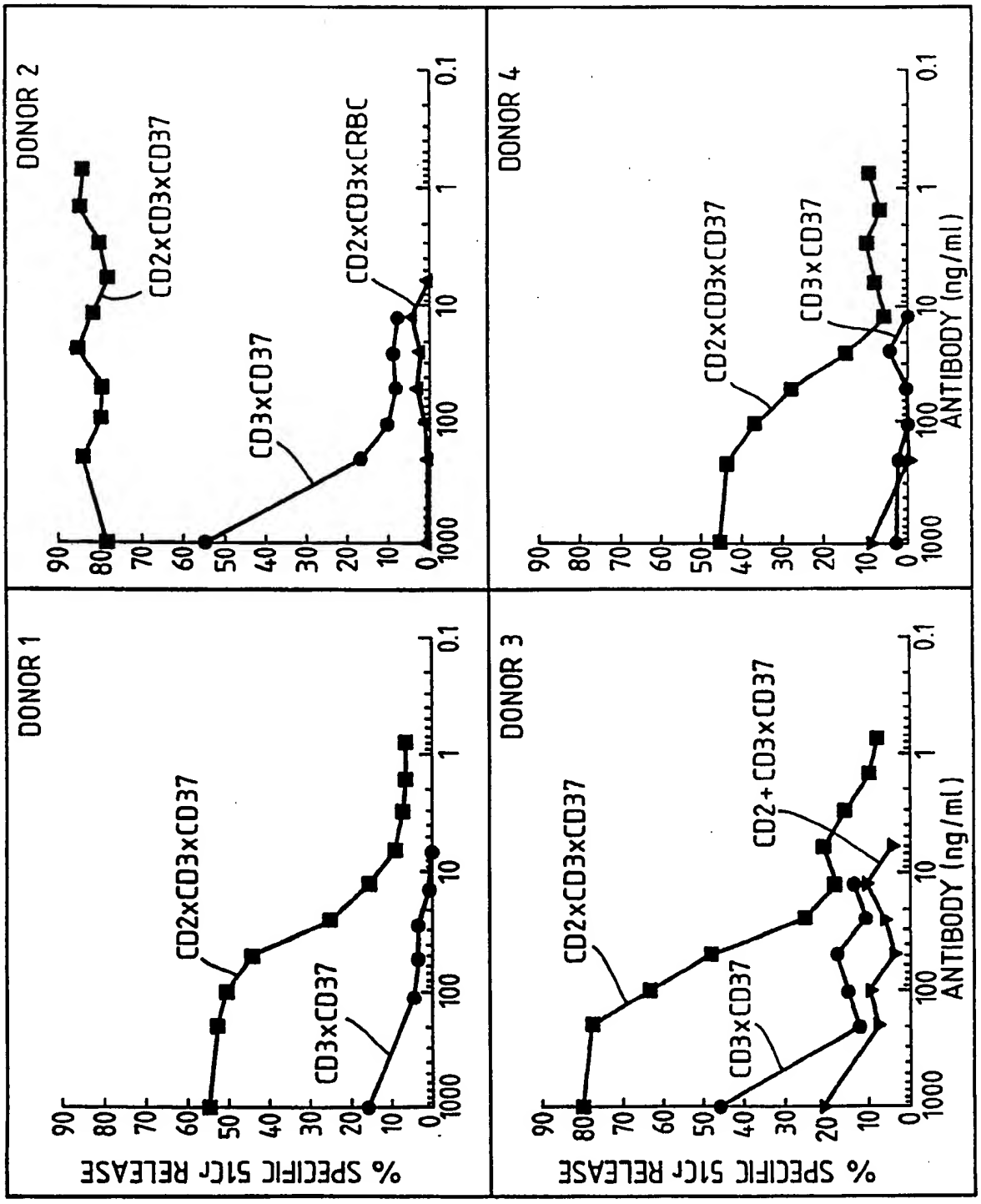
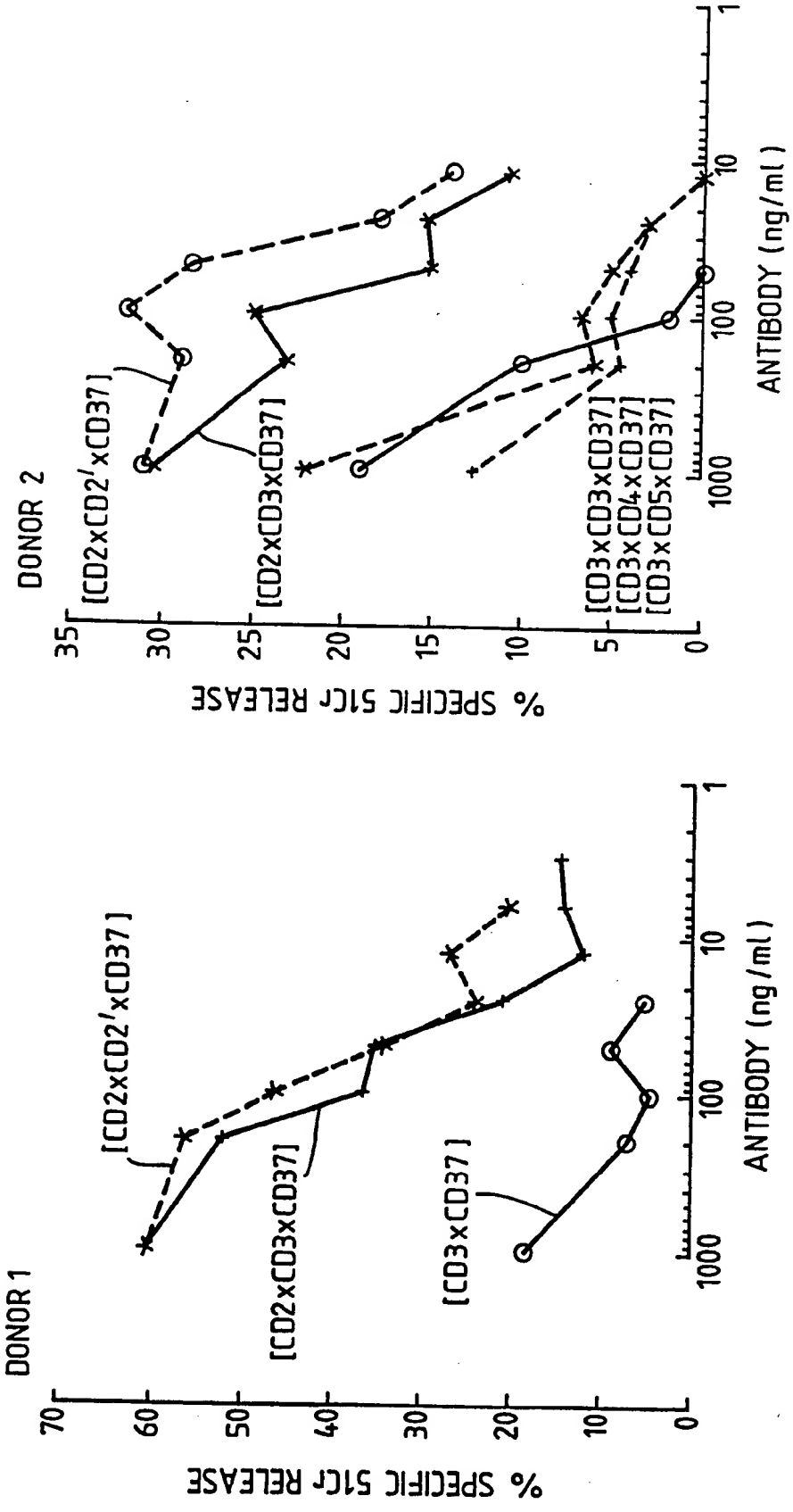

DONOR 1
DONOR 2
DONOR 3
DONOR 4
% SPECIFIC 51Cr RELEASE
ANTIBODY (ng/ml)
CD2xCD3xCD37
CD3xCD37
CD2xCD3xCRBC
CD2+CD3xCD37
0
10
20
30
40
50
60
70
80
90
0.1
1
10
100
1000

Fig. 12.

Fig. 13.


DONOR 1
% SPECIFIC 51Cr RELEASE
70
60
50
40
30
20
10
0
[CD2xCD2'xCD37]
[CD2xCD3xCD37]
[CD3xCD37]
1000
100
10
1
ANTIBODY (ng/ml)
DONOR 2
% SPECIFIC 51Cr RELEASE
35
30
25
20
15
10
5
0
[CD2xCD2'xCD37]
[CD2xCD3xCD37]
[CD3xCD3xCD37]
[CD3xCD4xCD37]
[CD3xCD5xCD37]
1000
100
10
1
ANTIBODY (ng/ml)

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01335

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/28, G 01 N 33/563, H 61 K 39/395, C 12 P21/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO, A1, 9001339 (IMMUNOMEDICS, INC.) 22 February 1990, see page 7 - page 14	1-11,15-32
P,X	EP, A2, 0336379 (ONCOGEN LTD) 11 October 1989, see the whole document	1-7,11,15
P,X	WO, A1, 9004413 (RESEARCH EXPLOITATION LIMITED) 3 May 1990, see page 22 - page 25	23-30
P,X	WO, A1, 8911863 (GLENNIE, MARTIN, JOHN) 14 December 1989, see the whole document	12-14

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th November 1990	11. 12. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	miss T. MORTENSEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Cancer Detection and Prevention, Vol. 12, 1988, Ian G. Barr et al: "Retargeting of Cytolytic T Lymphocytes by Heteroaggregated (Bispecific) Antibodies ", see page 439 - page 450	1-3
Y		4-32
	--	
X	Chemical Abstracts, volume 107, no. 25, 21 December 1987, (Columbus, Ohio, US), Glennie, Martin J et al: "Preparation and performance of bispecific F (ab'y)2 antibody containing thioether-linked Fab'y fragments ", see page 597, abstract 234429b, & J. Immunol. 1987, 139(7), 2367-2375	1-3
Y		4-32
	--	
X	EP, A2, 0294703 (DANA-FARBER CANCER INSTITUTE, INC.) 14 December 1988, see page 4 - page 10	1-3
Y		4-32
	--	
X	EP, A2, 0241907 (THE GENERAL HOSPITAL CORPORATION) 21 October 1987, see the whole document	1-3
	--	
A	Proc.Natl.Acad.Sci., Vol. 86, May 1989, J M Rojo et al: "Physical association of CD4 and the T-cell receptor can be induced by anti-T-cell receptor antibodies ", see page 3311 - page 3315	4-32
	--	
X	Blood, Vol. 74, No. 2, 1989, Albertus W. Wognum et al: "An Enzyme-Linked Immunosorbent Assay for Erythropoietin Using Monoclonal Antibodies, Tetrameric Immune Complexes, and Substrate Amplification ", see page 622 - page 628	1-3
	--	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FRC M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Journal of Immunological Methods, Vol. 120, 1989, Terry E. Thomas et al: "Specific binding and release of cells from beads using cleavable tetrameric antibody complexes ", see page 221 - page 231	1-3
Y	EP, A2, 0180171 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 7 May 1986, see claims	1-3,5-7, 31,32

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/01335

SA 39700

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 01/11/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9001339	22/02/90	AU-D-	4062889	05/03/90
		EP-A-	0353960	07/02/90
		US-A-	4925648	15/05/90
EP-A2- 0336379	11/10/89	AU-D-	3242389	05/10/89
		JP-A-	1304356	07/12/89
WO-A1- 9004413	03/05/90	NONE		
WO-A1- 8911863	14/12/89	AU-D-	3752089	05/01/90
EP-A2- 0294703	14/12/88	JP-A-	64003128	06/01/89
EP-A2- 0241907	21/10/87	JP-T-	2500321	08/02/90
		WO-A-	87/06240	22/10/87
EP-A2- 0180171	07/05/86	JP-A-	61234779	20/10/86

US005591828A

United States Patent [19]

Bosslet et al.

[11] Patent Number: 5,591,828

[45] Date of Patent: Jan. 7, 1997

[54] **BISPECIFIC AND OLIGOSPECIFIC MONO-AND OLIGOVALENT RECEPTORS, THE PREPARATION AND USE THEREOF**

[75] Inventors: **Klaus Bosslet**; **Peter Hermentin**; **Gerhard Seemann**, all of Marburg; **Ludwig Kuhlmann**, Flörsheim am Main; **Axel Steinsträsser**, Liederbach, all of Germany

[73] Assignee: **Behringwerke Aktiengesellschaft**, Marburg, Germany

[21] Appl. No.: **317,612**

[22] Filed: **Sep. 29, 1994**

Related U.S. Application Data

[63] Continuation of Ser. No. 147,428, Nov. 5, 1993, abandoned, which is a continuation of Ser. No. 17,439, Feb. 12, 1993, abandoned, which is a continuation of Ser. No. 541,020, Jun. 20, 1990, abandoned.

[30] **Foreign Application Priority Data**

Jun. 22, 1989 [DE] Germany 39 20 358.1

[51] **Int. Cl.**[6] **C07K 16/46**; C07K 16/28

[52] **U.S. Cl.** **530/387.3**; 435/188.5; 530/389.7; 530/388.8

[58] **Field of Search** 435/69.6; 530/387.3, 530/395, 388.8; 424/133.1, 155.1

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,652,440	3/1987	Paik et al.	424/1.1
4,714,681	12/1987	Reading	435/240.27

FOREIGN PATENT DOCUMENTS

0141079A3	5/1985	European Pat. Off. .
0369566A2	5/1990	European Pat. Off. .
369566	5/1990	European Pat. Off. .
WO86/01407	3/1986	WIPO .

OTHER PUBLICATIONS

H. Paulus, Behring Inst. Mitt. 78, (1985) 118–132.
U. D. Staerz and M. J. Bevan, Proc. Natl. Acad. Sci. USA 83, (1986) 1453–1457.
U. Zimmerman, Rev. Physio. Biochem. Pharmacol 105 (1986), 176–260.
J. Van Duk et al., Int. J. Cancer 43, (1989) 344–349.
M. W. Brechbiel et al., Inorganic Chemistry 25, (1986) 2772–2781.
Shulman et al., Nature 276, (1978) 269.
Cordell et al., J. Histochem. Cytochem. 32, (1984) 219.
Molecular Cloning, A Laboratory Manual; Sambrook, Fritsch, Maniatis; Cold Spring Harbor Laboratory, 1982, (S. 11–44, 51–127, 133–134, 141, 146, 150–167, 170, 188–193, 197–199, 248–255, 270–294, 310–328, 364–401, 437–506).
Molecular Cloning, A Laboratory Manual; Sambrook, Fritsch, Maniatis; Cold Spring Harbor Laboratory Press, 1989, (S. 16.2–16.22, 16.30–16.40, 16.54–16.55).
A. M. Frischauf et al., J. Mol. Biol. 170, (1983) 827–842.
G. H. A. Seeman et al., The EMBO Journal 5, (1986) 547–552.
Orlandi et al., Proc. Natl. Acad. Sci., USA 86, (1989) 3833–3837.
Simon et al., Nucl. Acids Res. 16, (1988) 354.
P. L. Ey et al., Immunochemistry 15, (1978) 429.
E. K. O'Shea et al., Science 245, (1989) 646–648.
J. P. Tam et al., J. Am. Chem. Soc. 105, (1983) 6442–6455.
J. Rivier et al., J. Chromatography 288, (1984) 303–328.
Beidler et al., The Journal of Immunology 141(11): 4053–60 (1988).
Williams, Trends in Biotechnology 6(2): 36–39, 42 (1988).
V. T. Oi & S. L. Morrison, Biotechnique 4(3): 214–21 (1986).
Tao et al. Journal of Immunology vol. 143 p. 2595 1989.
Osbund et al. Immunology Today, vol. 11, No. 6 p. 193 1990.
Waldmann, Science vol. 252 p. 1657 1991.
Harris et al. TibTech vol. 11 p. 42 1993.
Stanfield et al. TibTech vol. 12 1994 p. 275.
Hird et al. Genes and Cancer p. 183, 1990 ed. Carney and Sikora.
Lazar et al. Molecular and Cellular Biology p. 1247, 1988.
Gillies et al. Human Antibodies & Hybridomers 1(1) 1990 p.47.
Burgess et al. Journal of Cell Biology vol. 111 1990 p. 2129.
Huston et al. PNAS. vol. 85 5879, 1988.
Titus et al Journal of Immunology vol. 139 3153 N1 1987.

Primary Examiner—Lila Feisee
Attorney, Agent, or Firm—Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.

[57] **ABSTRACT**

The invention relates to bispecific and oligospecific, mono- and oligovalent receptors which are prepared by gene manipulation by fusion of DNA coding for F(ab) fragments of antibodies of two or more different specificities by means of suitable linkers. In this connection, one specificity is preferably directed either against an epitope, which is located on the cell membrane or in the interstitium, of a tumor-associated antigen (TAA) or against an epitope in the tumor endothelium (TE), while the other specificities relate to high-molecular or low-molecular weight ligands and react, for example, with the Komplexons ethylenediaminetetraacetate and diethylenetriaminepentaacetate in Y90 complexed form (EDTA-Y90 and DTPA-Y90 respectively). In a particularly preferred embodiment, the binding with the Komplexons takes place on the Komplexon receptor arm via fos-jun interaction (or else avidin-biotin interaction). Other preferred specificities have catalytic properties.

10 Claims, 22 Drawing Sheets

FIG. 1

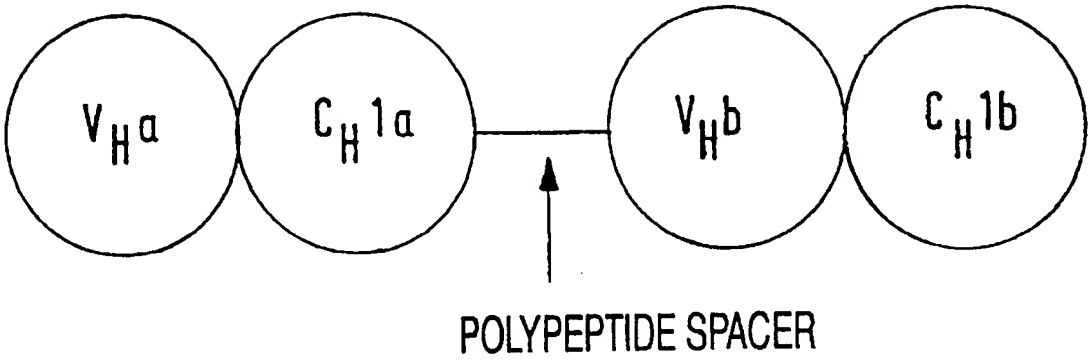
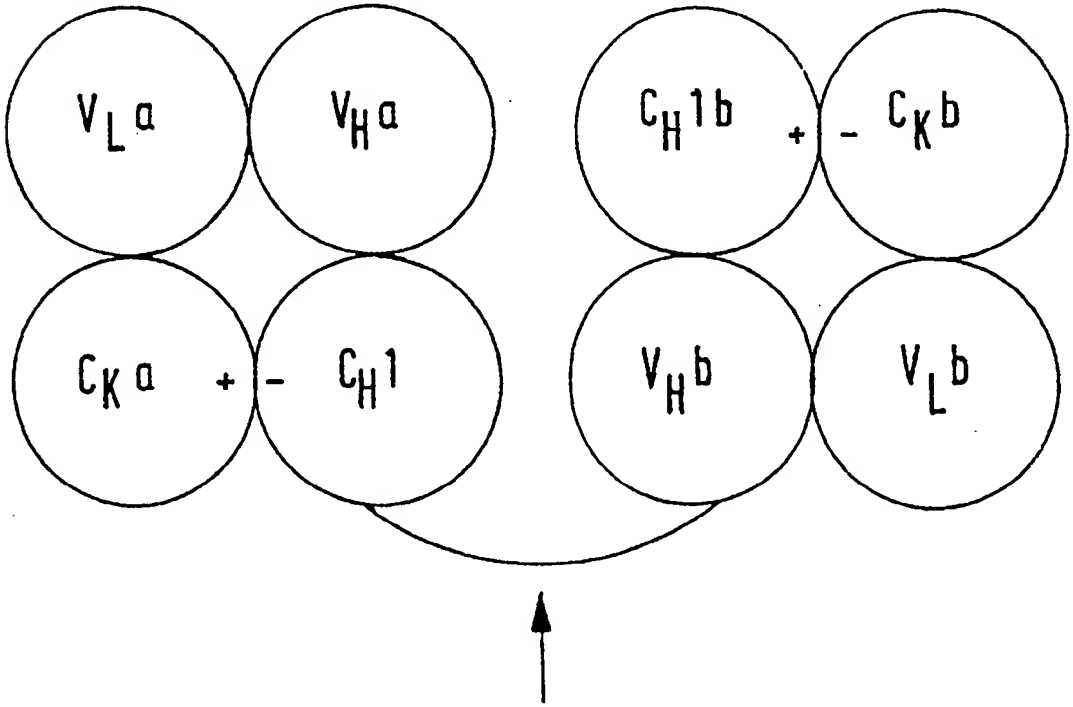

$V_H a$
$C_H 1a$
$V_H b$
$C_H 1b$
POLYPEPTIDE SPACER

FIG. 2


$V_L a$
$V_H a$
$C_H 1b$
+
-
$C_K b$
$C_K a$
+
-
$C_H 1$
$V_H b$
$V_L b$
POLYPEPTIDE SPACER

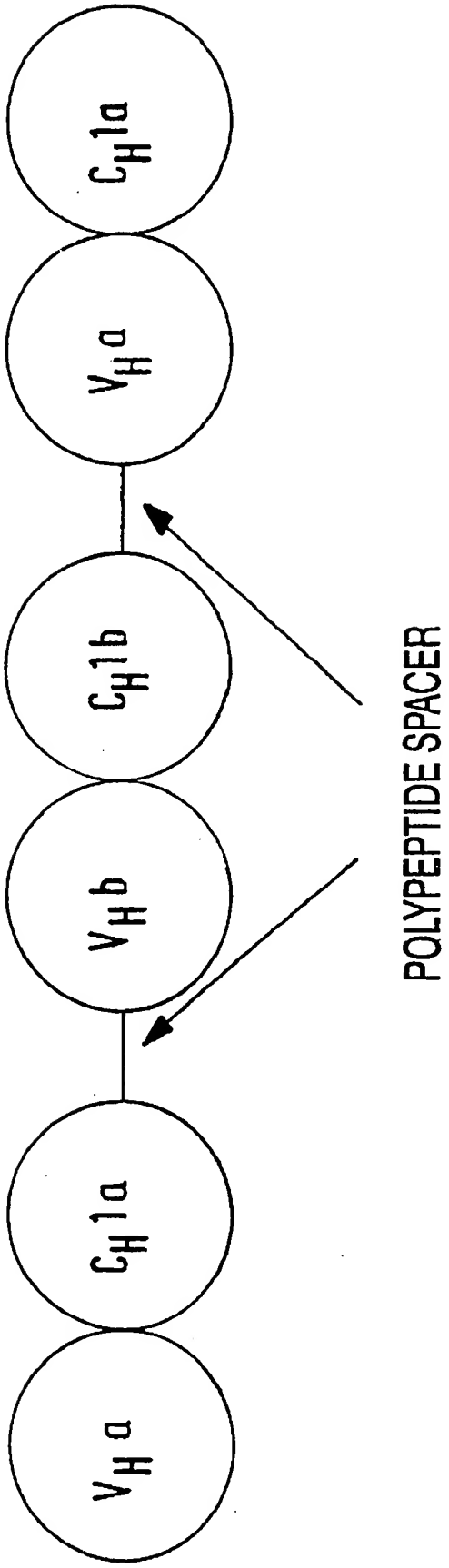


FIG. 3

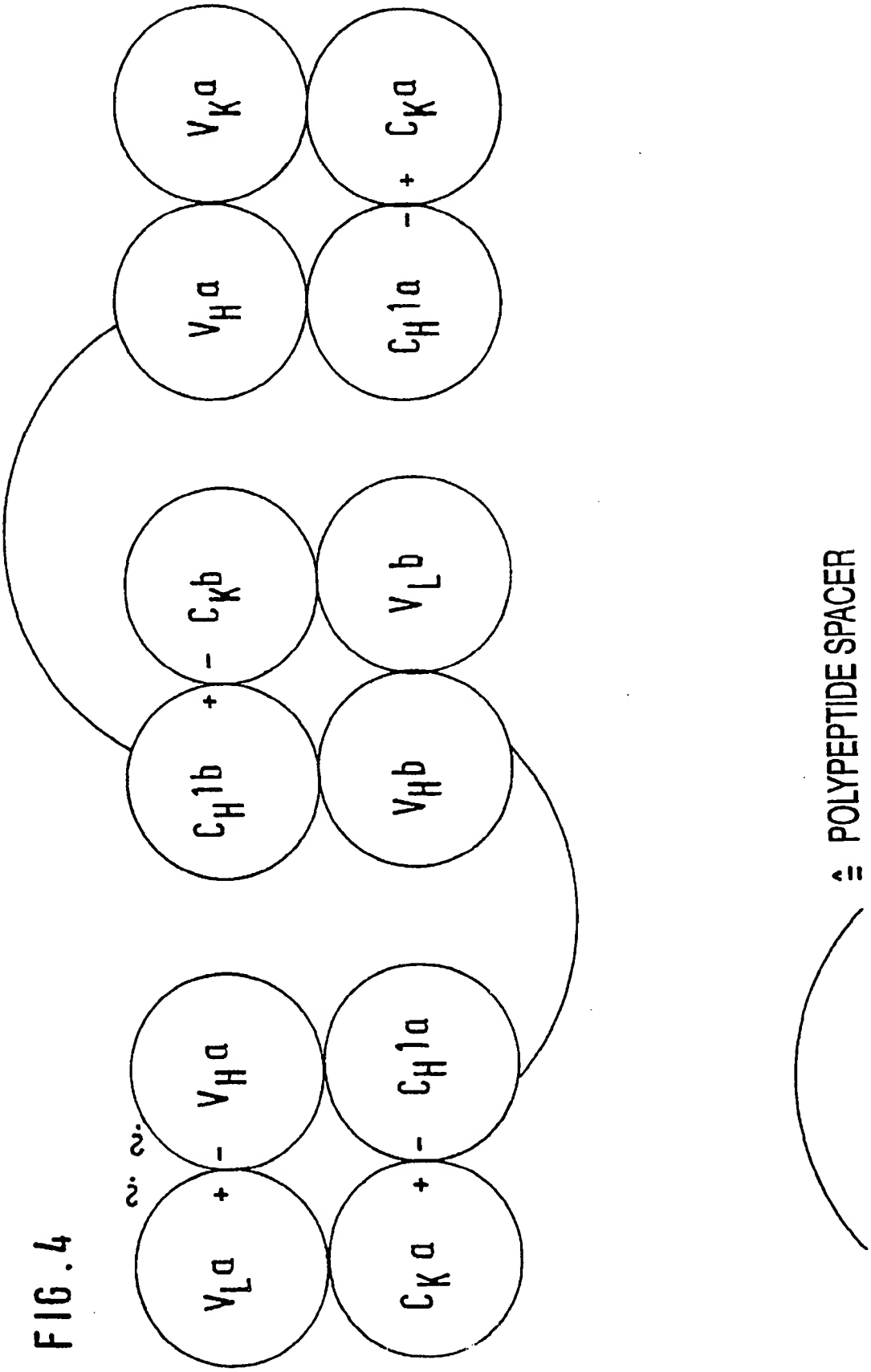

$V_K{}^a$
$C_K{}^a$
$V_H{}^a$
C_H1^a
$C_K{}^b$
$V_L{}^b$
C_H1^b
$V_H{}^b$
$V_H{}^a$
C_H1^a
$V_L{}^a$
$C_K{}^a$
= POLYPEPTIDE SPACER

FIG. 4

FIG. 5

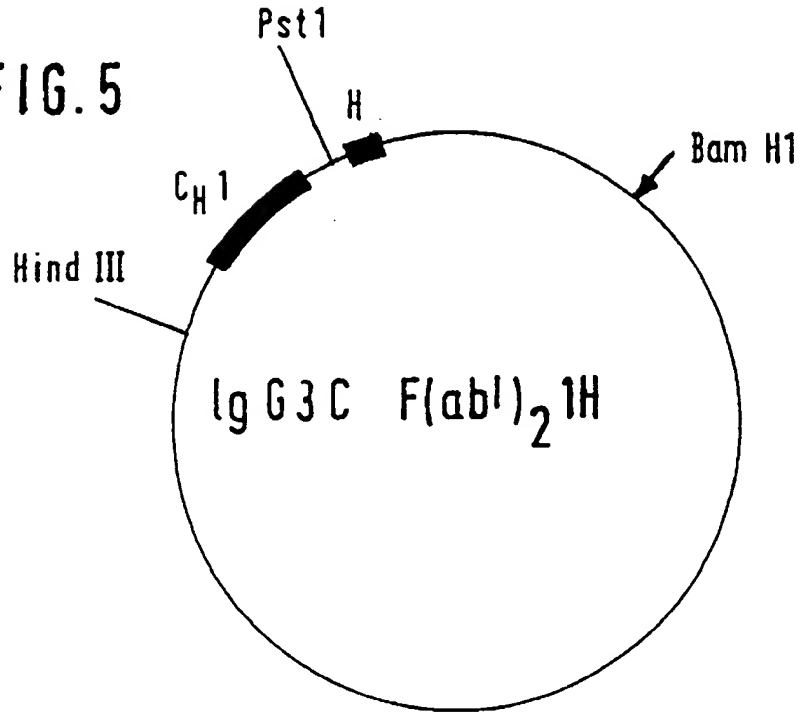

Pst1
H
Bam H1
C_H1
Hind III
lg G3C F(ab')$_2$1H

FIG. 6

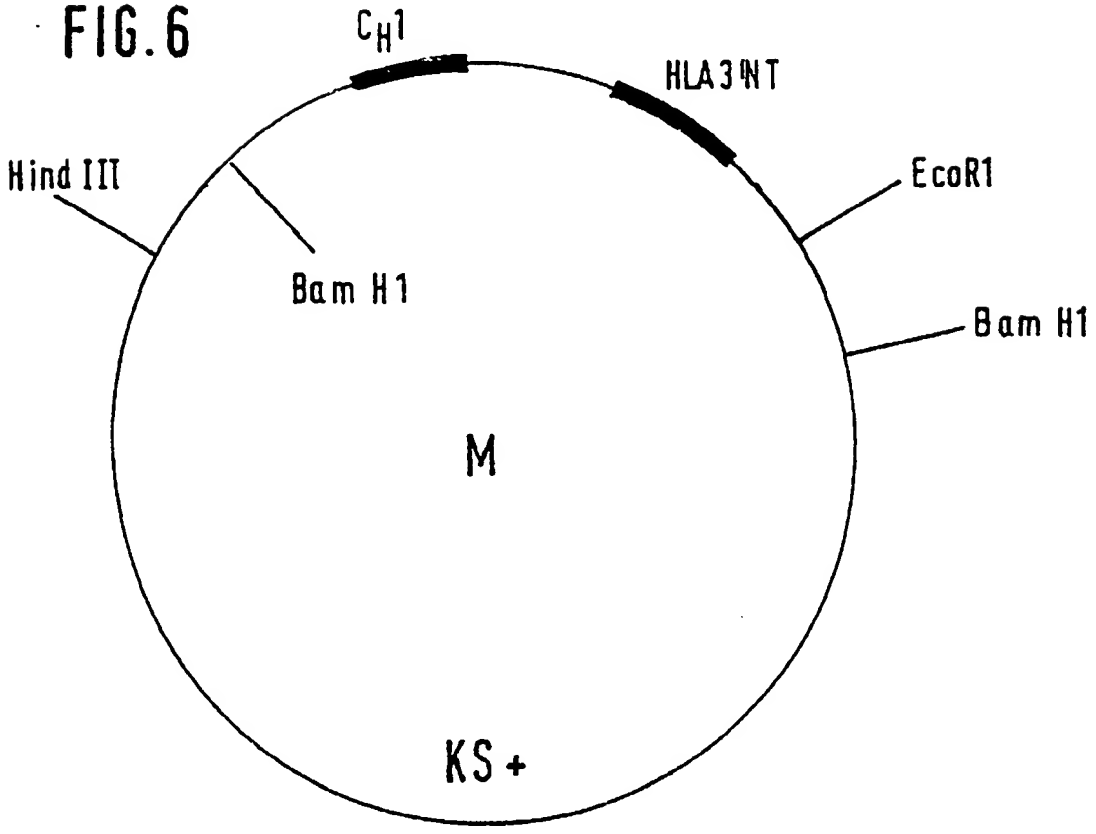

C_H1
HLA3NT
Hind III
EcoR1
Bam H1
Bam H1
M
KS+

FIG.7

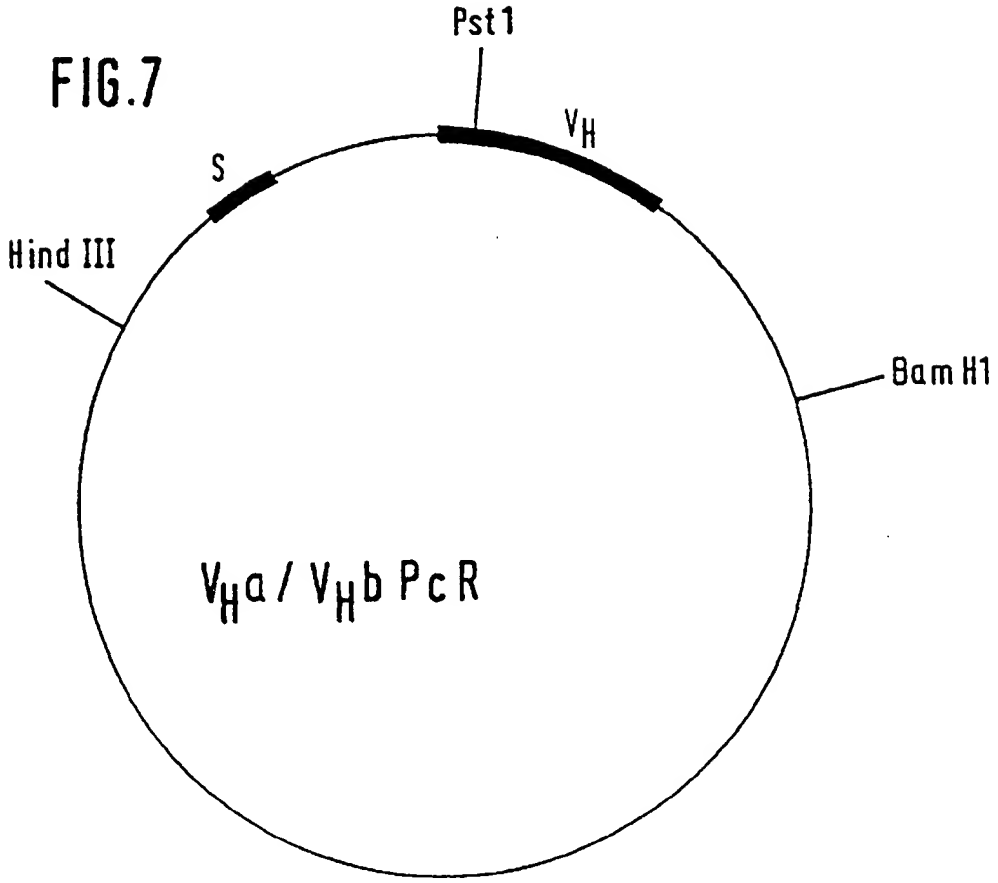
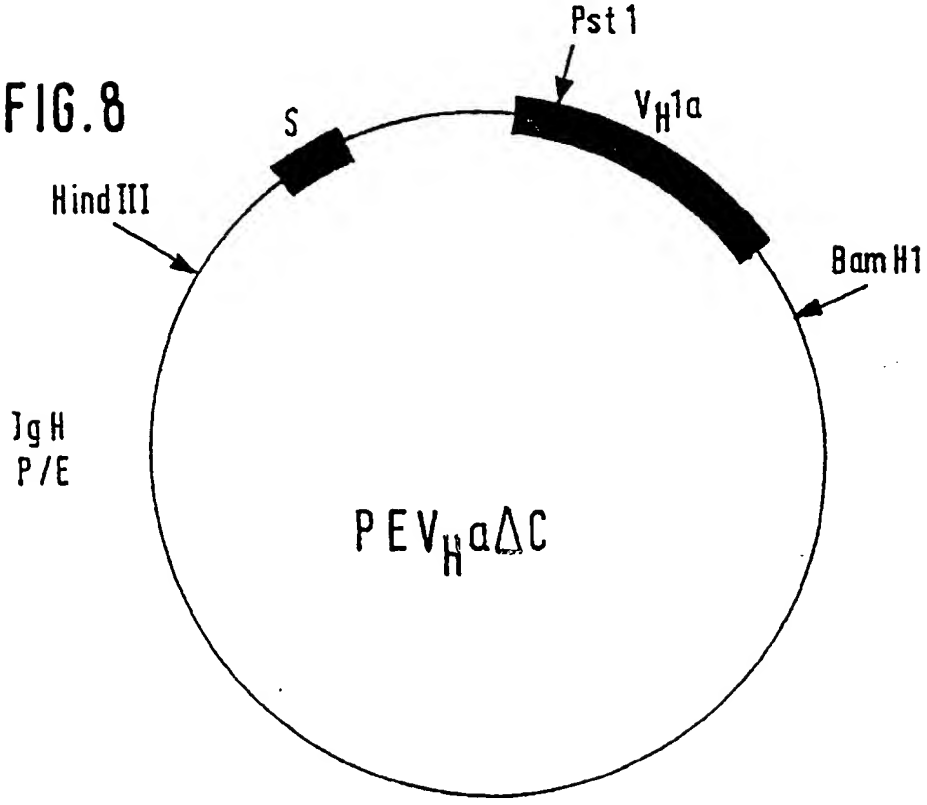

Pst1
V_H
S
Hind III
Bam H1
$V_H a / V_H b$ PcR

FIG.8


Pst 1
$V_H 1a$
S
Hind III
Bam H1
Jg H
P/E
PE$V_H a \Delta C$

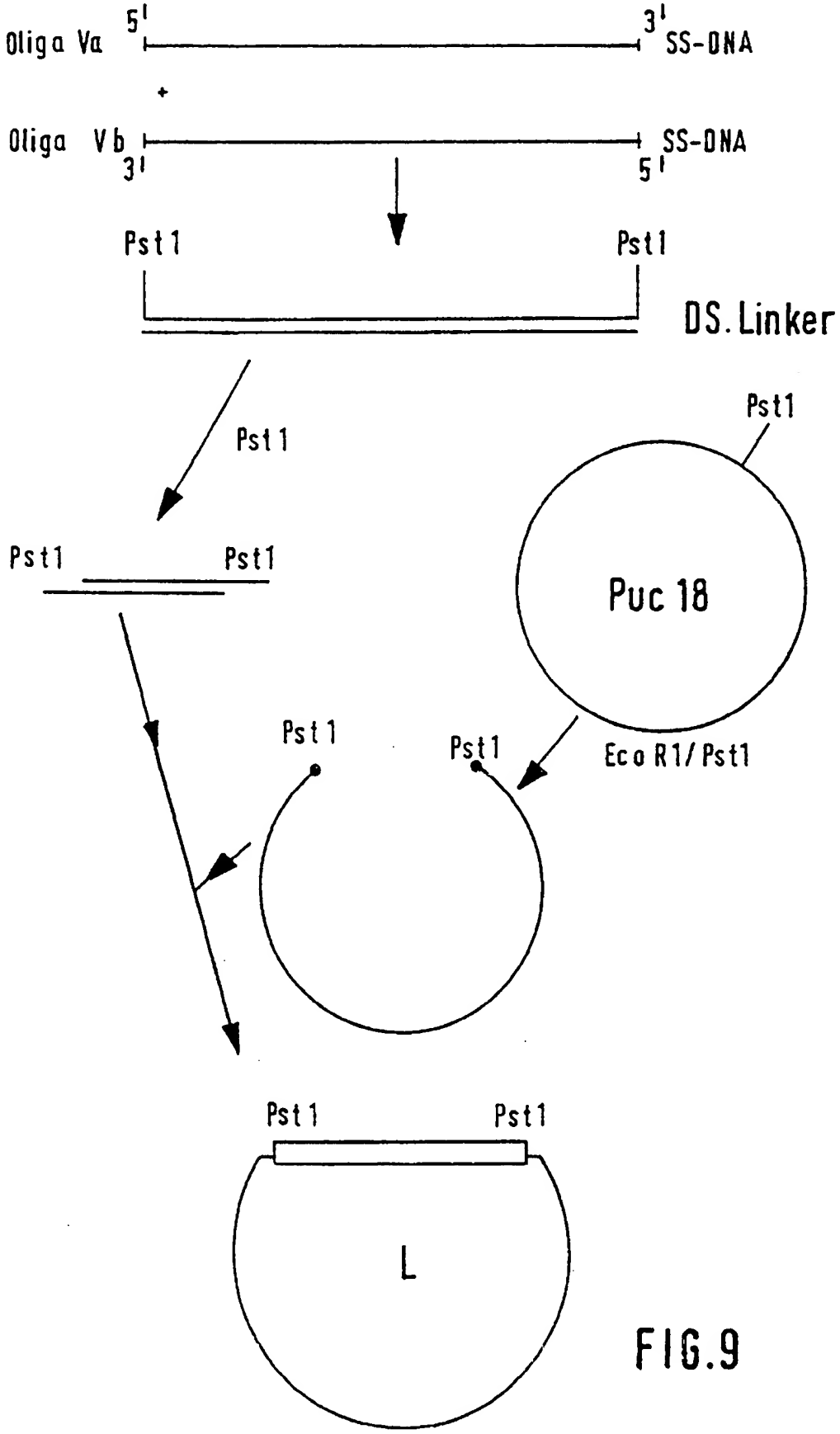

Oligo Va
5'
3'
SS-DNA
+
Oligo Vb
3'
5'
SS-DNA
Pst1
Pst1
DS. Linker
Pst1
Pst1
Pst1
Pst1
Puc 18
Pst1
Pst1
Eco R1/Pst1
Pst1
Pst1
L

FIG.9

FIG. 10

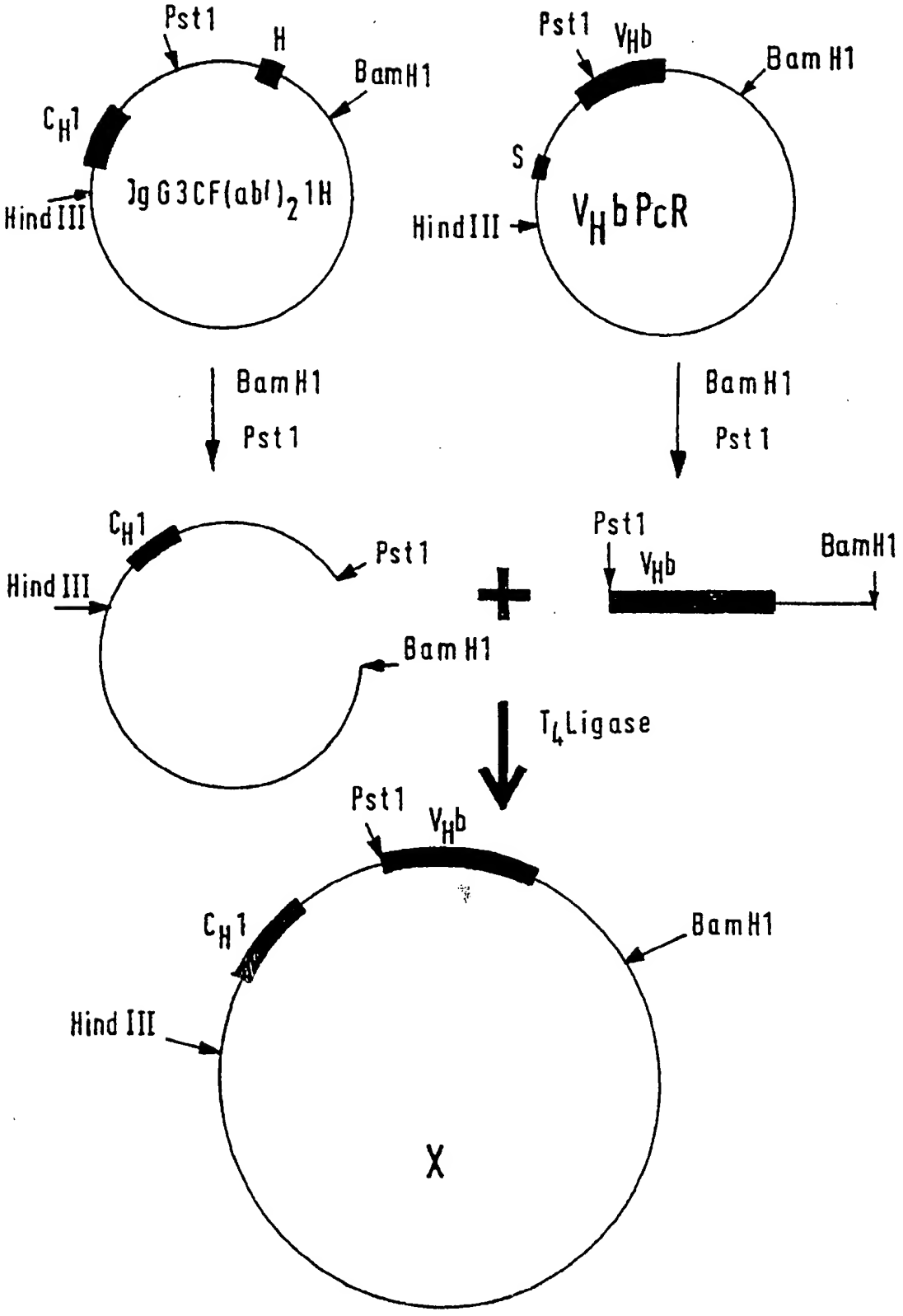

Pst1
H
BamH1
C_H1
Hind III
Jg G3CF(ab')$_2$1H
Pst1
V_{Hb}
Bam H1
S
Hind III
V_Hb PcR
BamH1
Pst1
BamH1
Pst1
C_H1
Pst1
Hind III
Bam H1
Pst1
V_{Hb}
BamH1
T_4Ligase
Pst1
V_{Hb}
C_H1
BamH1
Hind III
X

FIG. 11

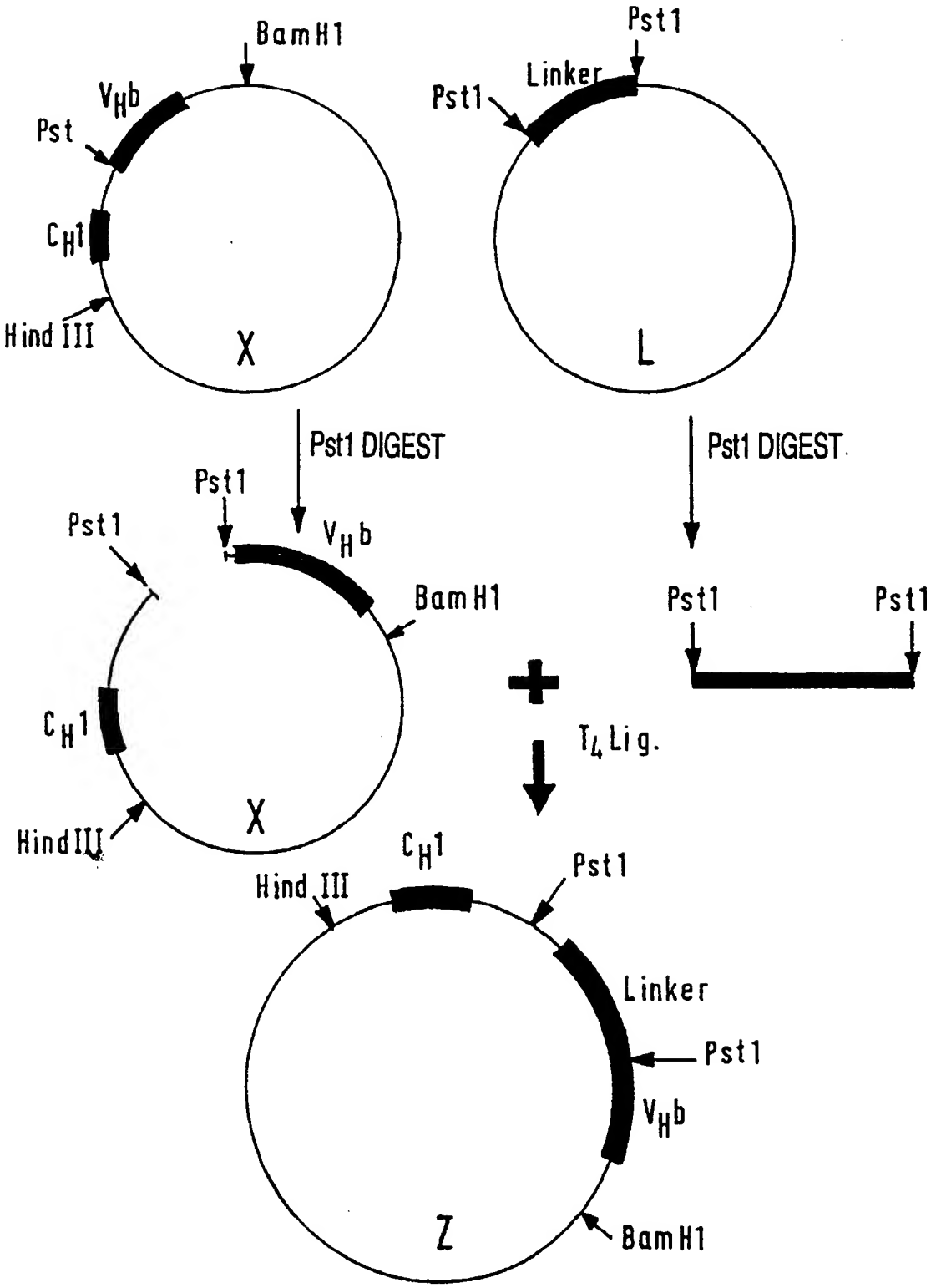

BamH1
V_Hb
Pst
C_H1
Hind III
X
Pst1
Linker
Pst1
L
Pst1 DIGEST
Pst1 DIGEST
Pst1
Pst1
V_Hb
BamH1
Pst1
Pst1
C_H1
Hind III
X
+
T_4 Lig.
C_H1
Pst1
Hind III
Linker
Pst1
V_Hb
BamH1
Z

FIG. 12

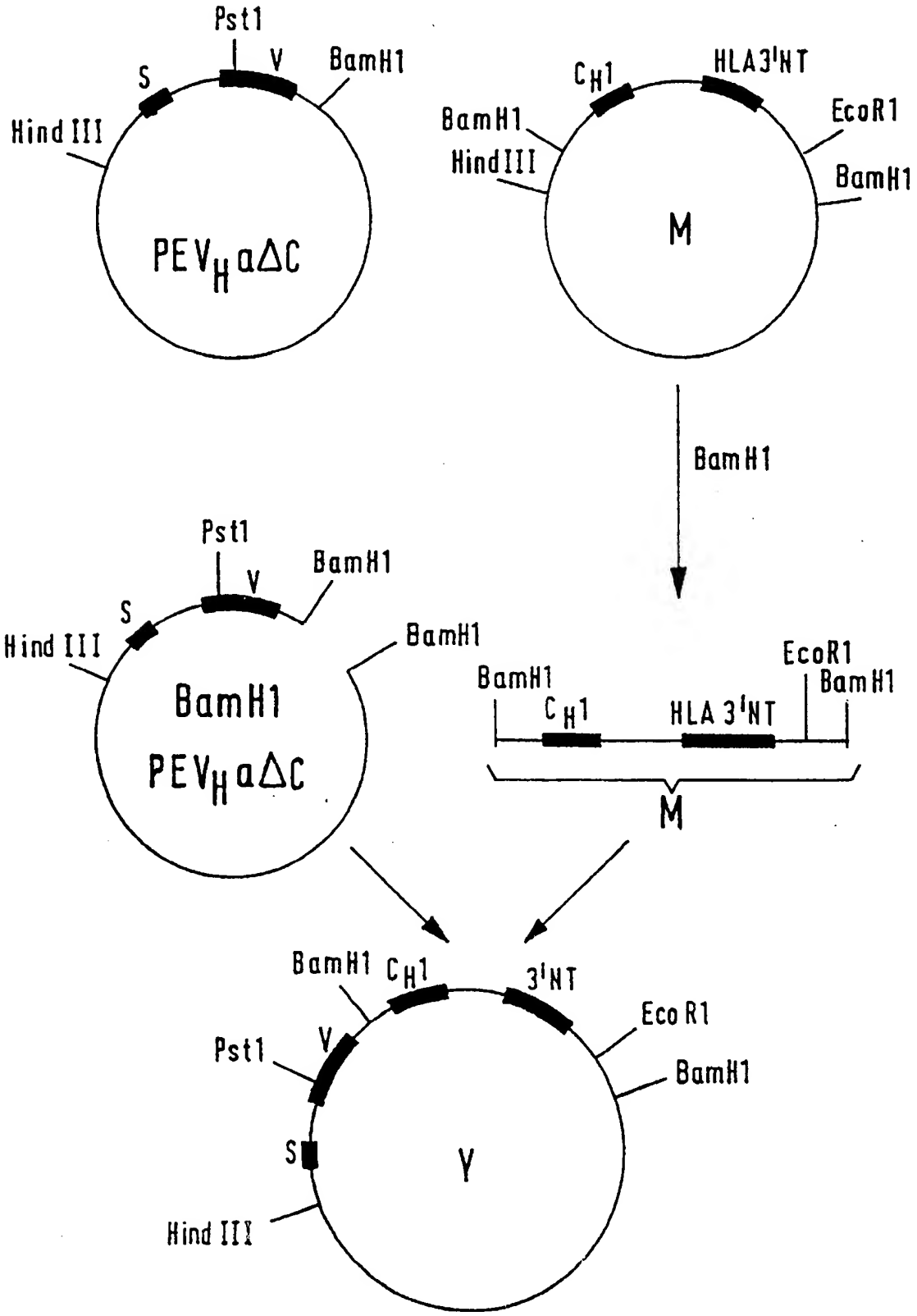

Pst1
V
BamH1
S
Hind III
PEV$_H$aΔC
C$_H$1
HLA3'NT
EcoR1
BamH1
HindIII
BamH1
M
BamH1
Pst1
V
BamH1
S
Hind III
BamH1
BamH1
PEV$_H$aΔC
EcoR1
BamH1
BamH1
C$_H$1
HLA 3'NT
BamH1
M
BamH1 C$_H$1
3'NT
Pst1
V
Eco R1
BamH1
S
Hind III
Y

FIG. 13

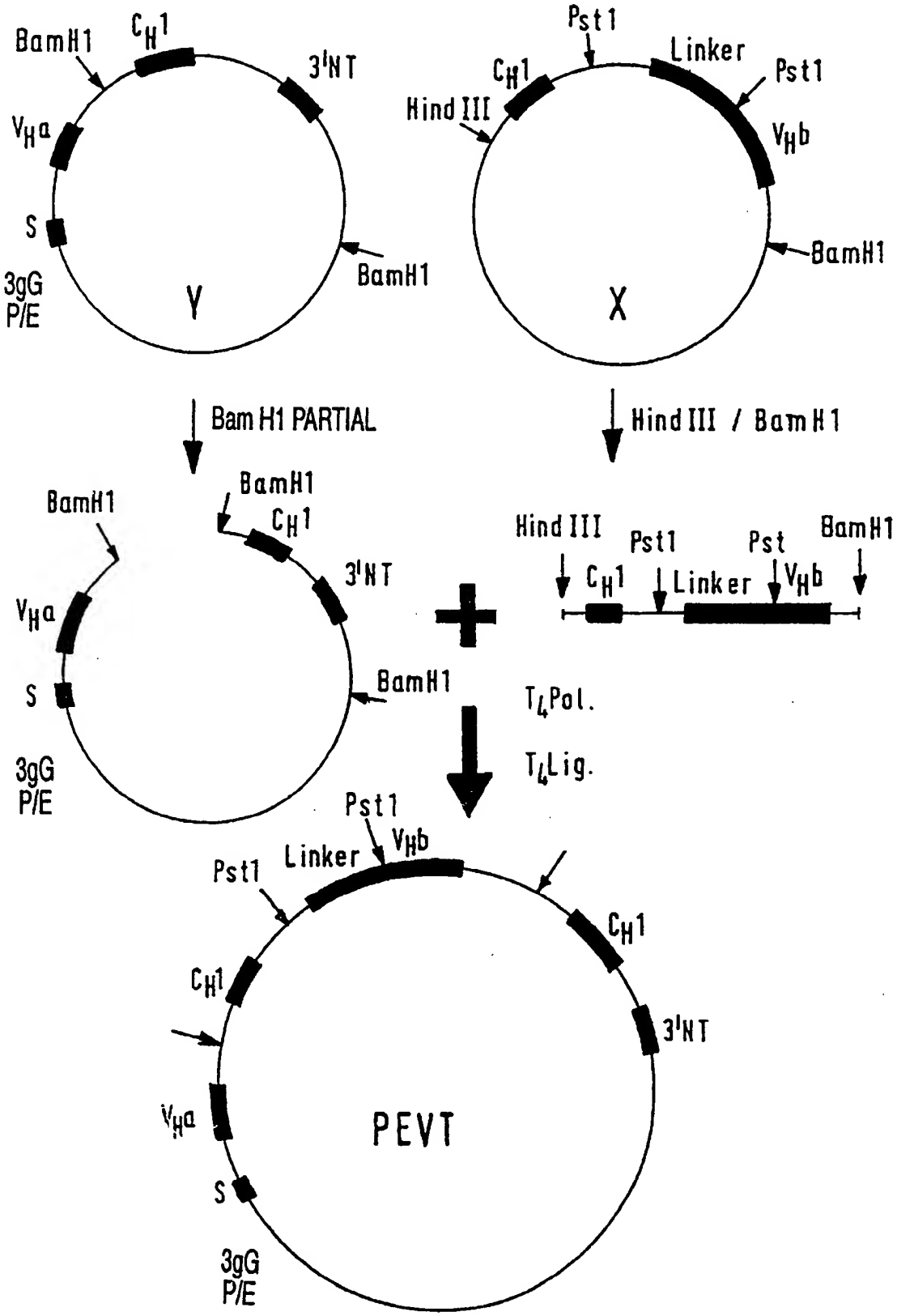

BamH1
C_H1
3'NT
V_Ha
S
3gG P/E
Y
BamH1
Pst1
Linker
Pst1
C_H1
Hind III
V_Hb
BamH1
X
Bam H1 PARTIAL
Hind III / Bam H1
BamH1
BamH1
C_H1
3'NT
V_Ha
S
3gG P/E
BamH1
Hind III
Pst1
Pst
BamH1
C_H1
Linker
V_Hb
T_4Pol.
T_4Lig.
Pst1
V_Hb
Linker
Pst1
C_H1
C_H1
3'NT
V_Ha
PEVT
S
3gG P/E

FIG.14

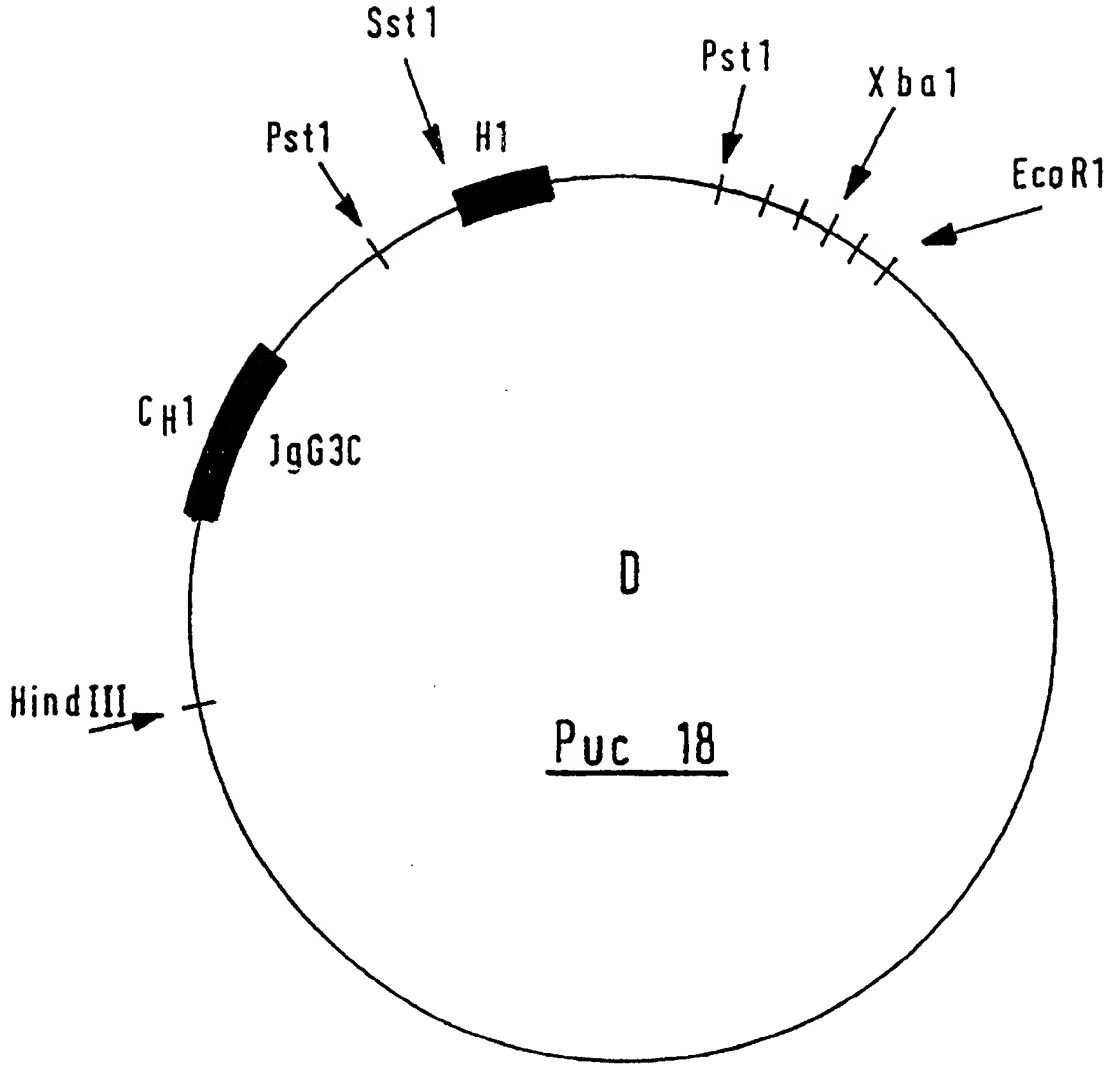


FIG. 15

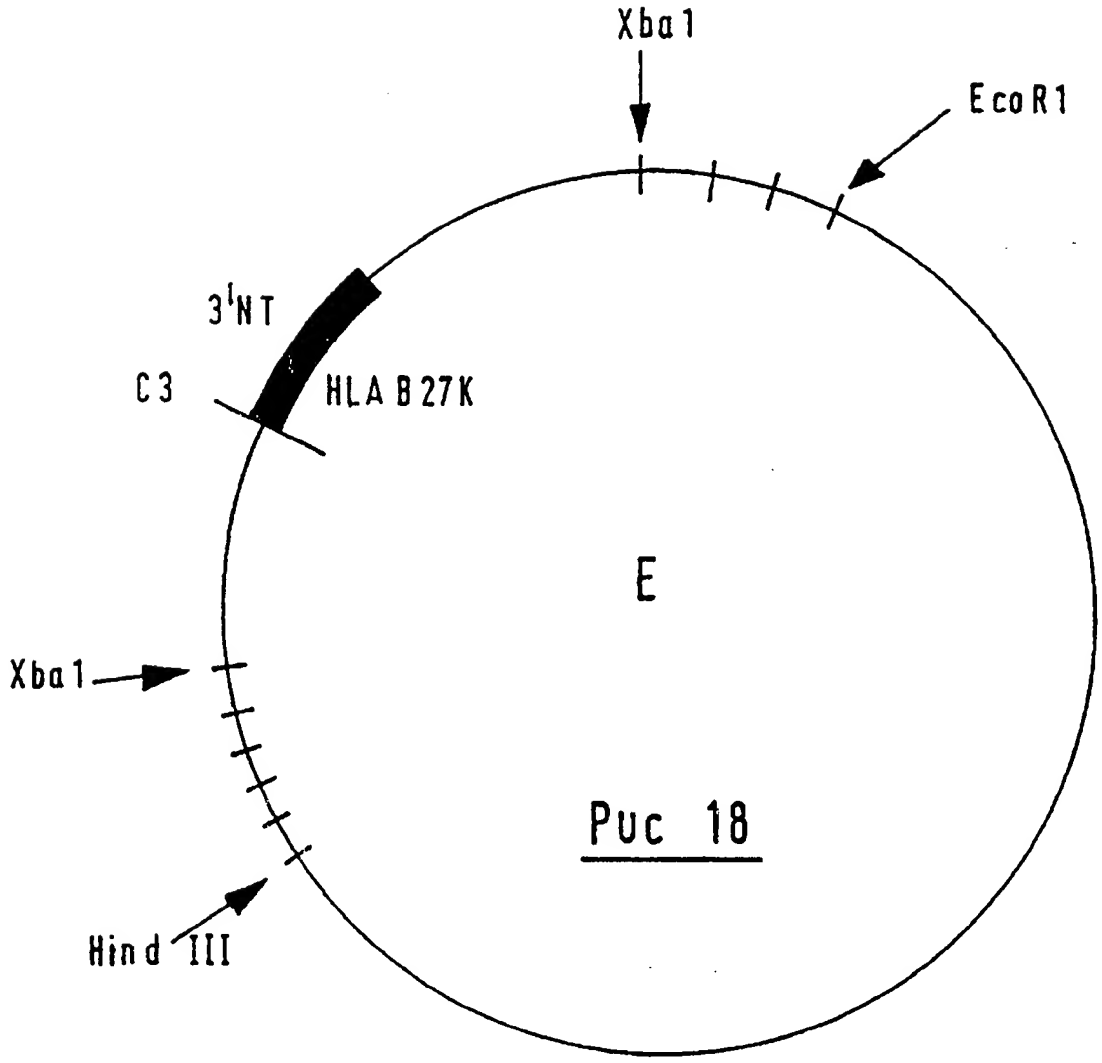


FIG.16

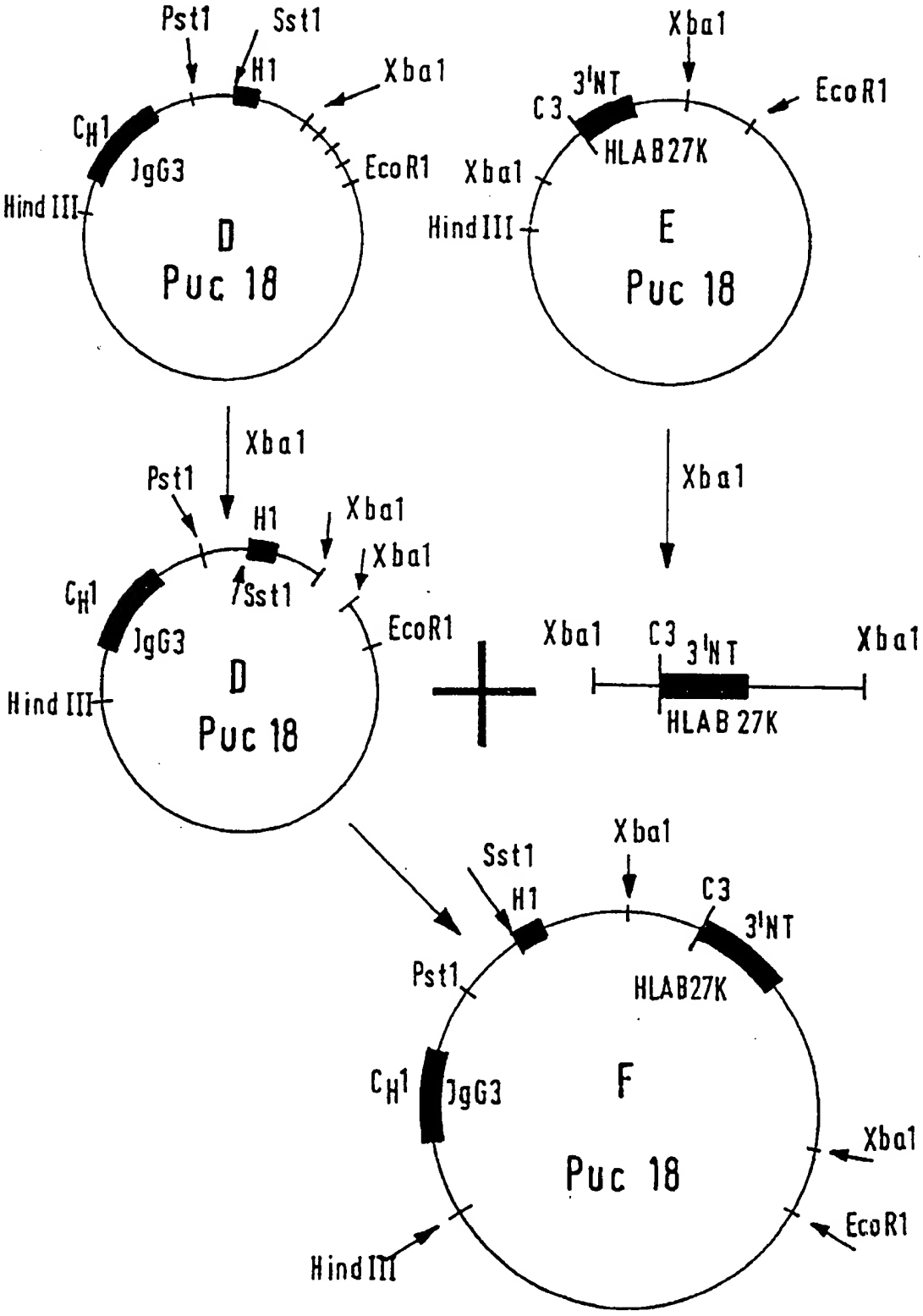

Pst1
Sst1
H1
Xba1
CH1
JgG3
EcoR1
Hind III
D
Puc 18
Xba1
3'NT
C3
EcoR1
HLAB27K
Xba1
Hind III
E
Puc 18
Xba1
Xba1
Pst1
H1
Xba1
Xba1
CH1
Sst1
EcoR1
JgG3
Hind III
D
Puc 18
Xba1
C3
3'NT
Xba1
HLAB 27K
Xba1
Sst1
H1
C3
3'NT
Pst1
HLAB27K
CH1
JgG3
F
Xba1
Puc 18
EcoR1
Hind III

FIG. 17

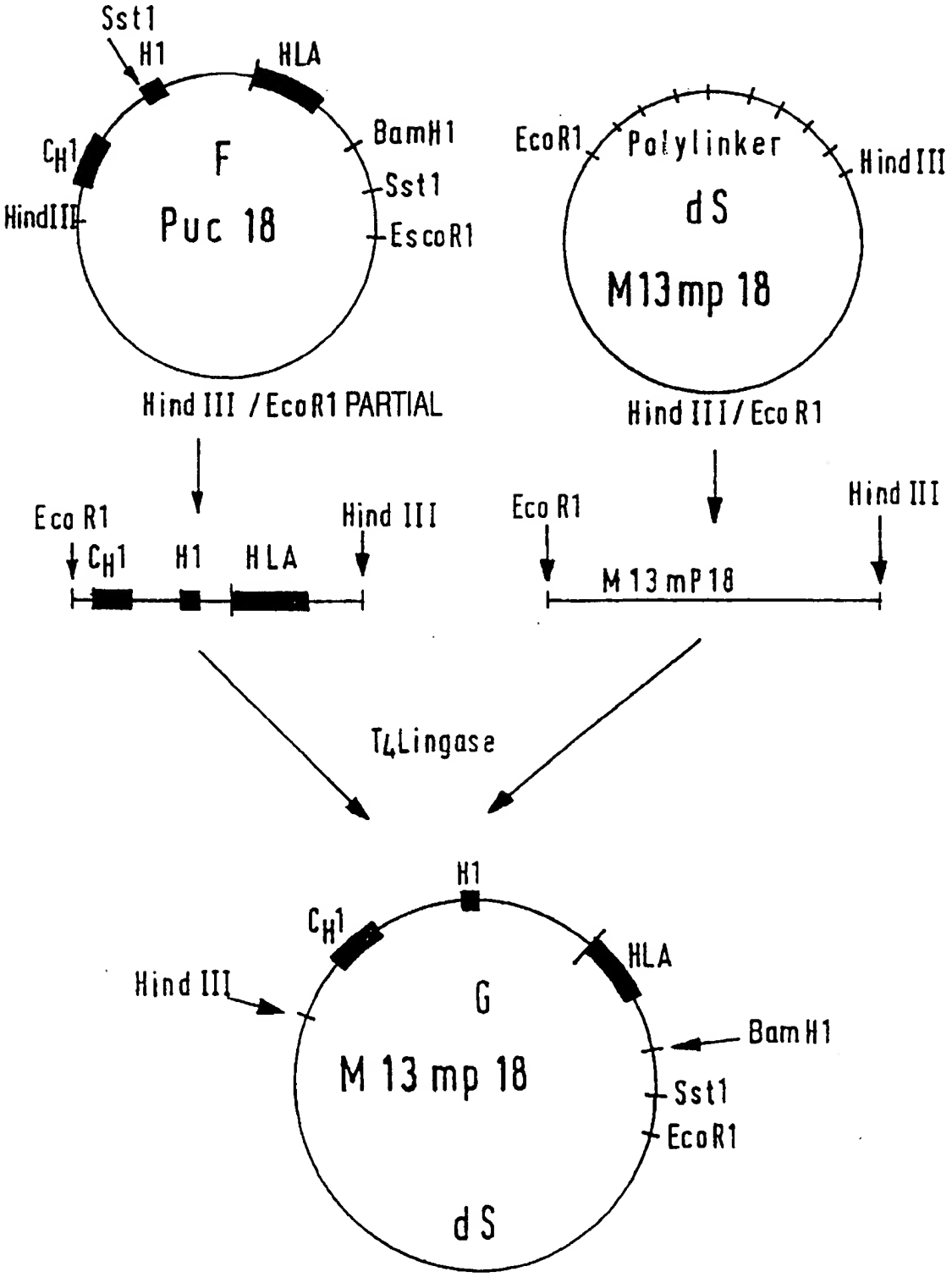

Sst1
H1
HLA
BamH1
Sst1
EscoR1
HindIII
CH1
F
Puc 18
EcoR1
Polylinker
HindIII
dS
M13mp 18
Hind III / EcoR1 PARTIAL
Hind III / Eco R1
Eco R1
Hind III
CH1
H1
HLA
Eco R1
Hind III
M 13 mP 18
T4Lingase
H1
CH1
HLA
Hind III
G
M 13 mp 18
BamH1
Sst1
EcoR1
dS

FIG. 18

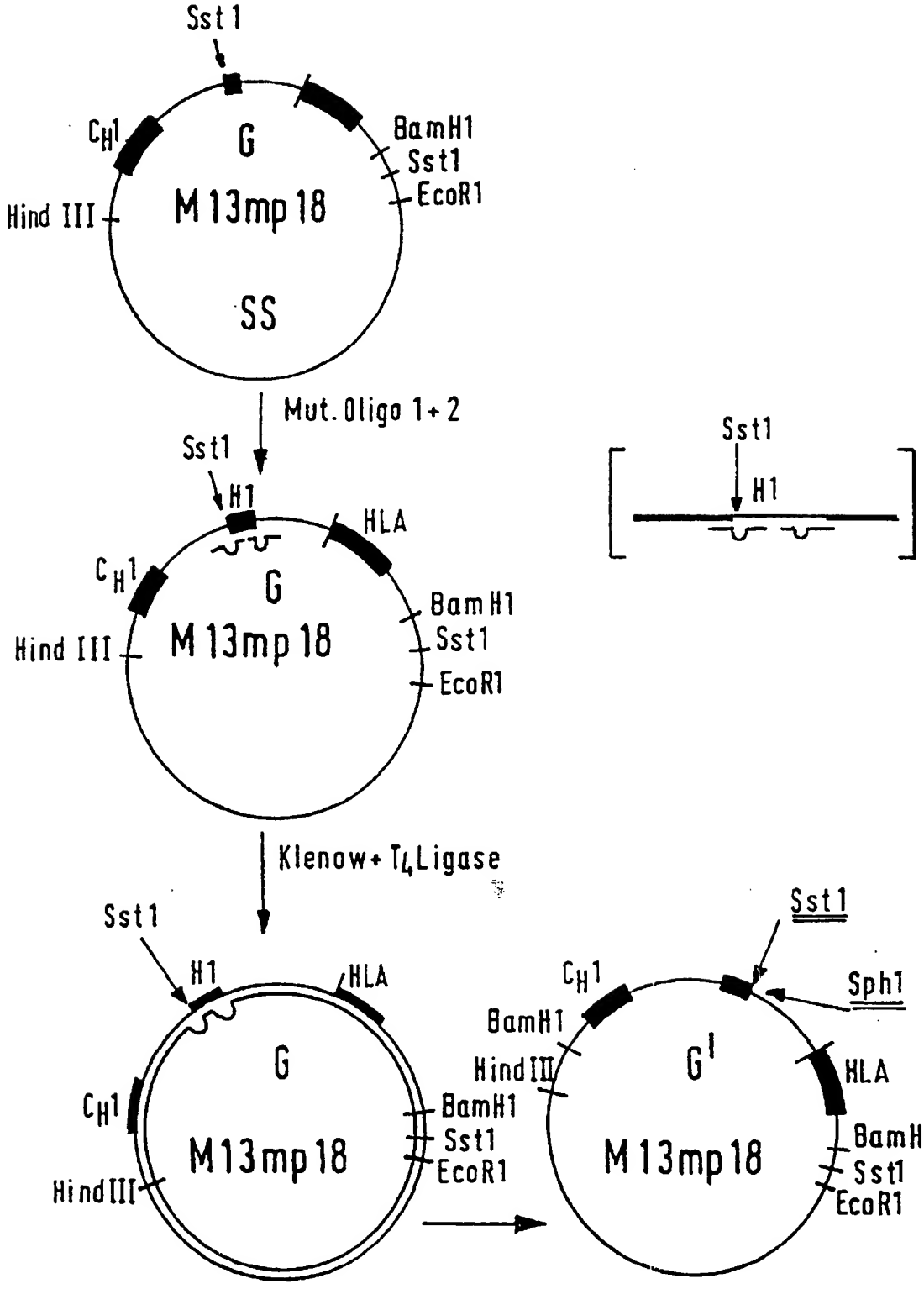

Sst1
CH1
G
BamH1
Sst1
EcoR1
Hind III
M13mp18
SS
Mut. Oligo 1+2
Sst1
H1
HLA
CH1
G
BamH1
Hind III
M13mp18
Sst1
EcoR1
Sst1
H1
Klenow+T4Ligase
Sst1
H1
HLA
G
CH1
BamH1
Sst1
EcoR1
HindIII
M13mp18
Sst1
CH1
Sph1
BamH1
HindIII
G'
HLA
BamH1
Sst1
EcoR1
M13mp18

FIG. 19

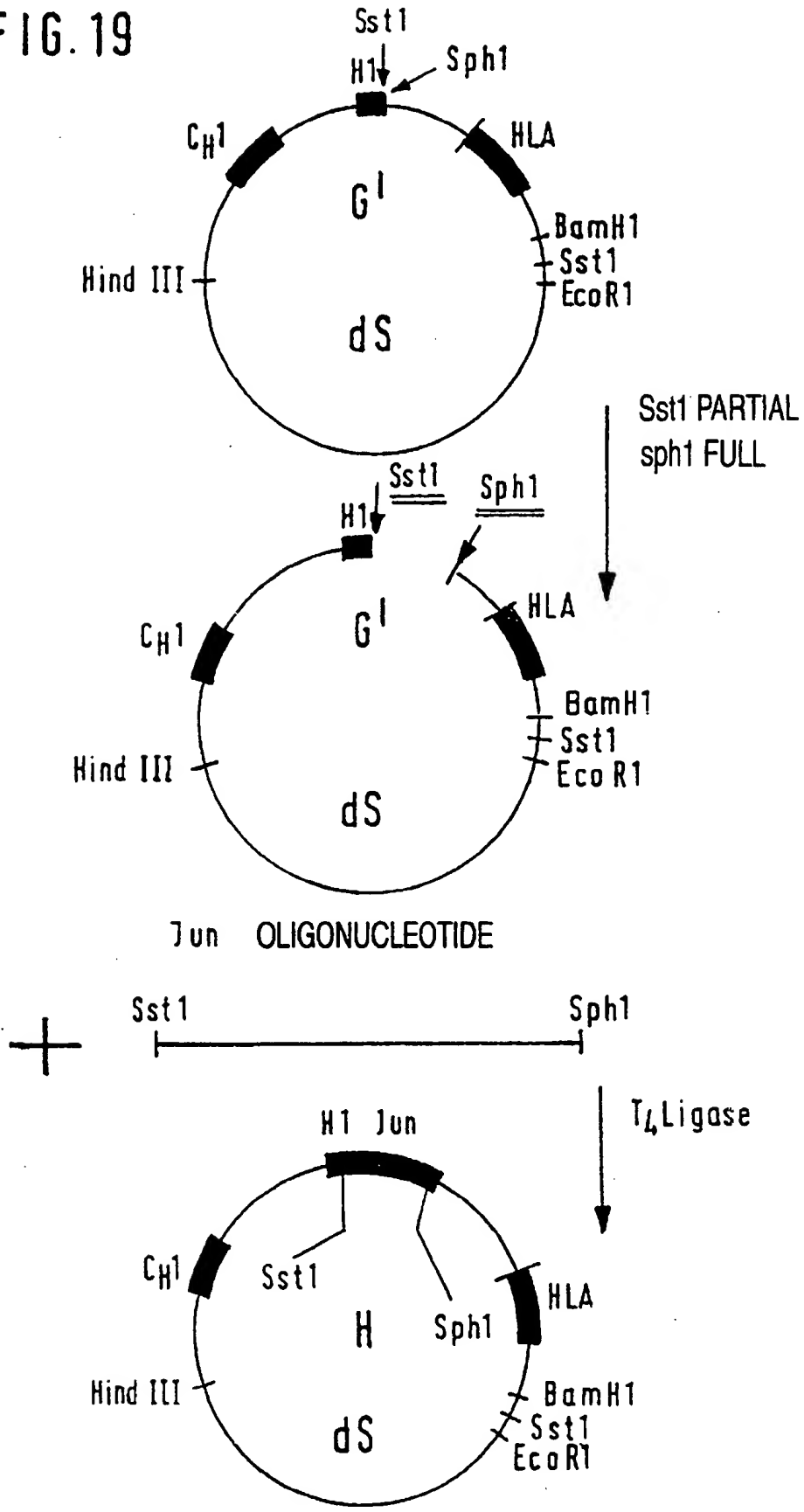

Sst1
H1
Sph1
C_H1
HLA
G
BamH1
Sst1
EcoR1
Hind III
dS
Sst1 PARTIAL
sph1 FULL
Sst1
Sph1
H1
C_H1
G
HLA
BamH1
Sst1
Eco R1
Hind III
dS
Jun OLIGONUCLEOTIDE
Sst1
Sph1
T_4Ligase
H1 Jun
C_H1
Sst1
H
Sph1
HLA
Hind III
BamH1
Sst1
EcoR1
dS

FIG. 20

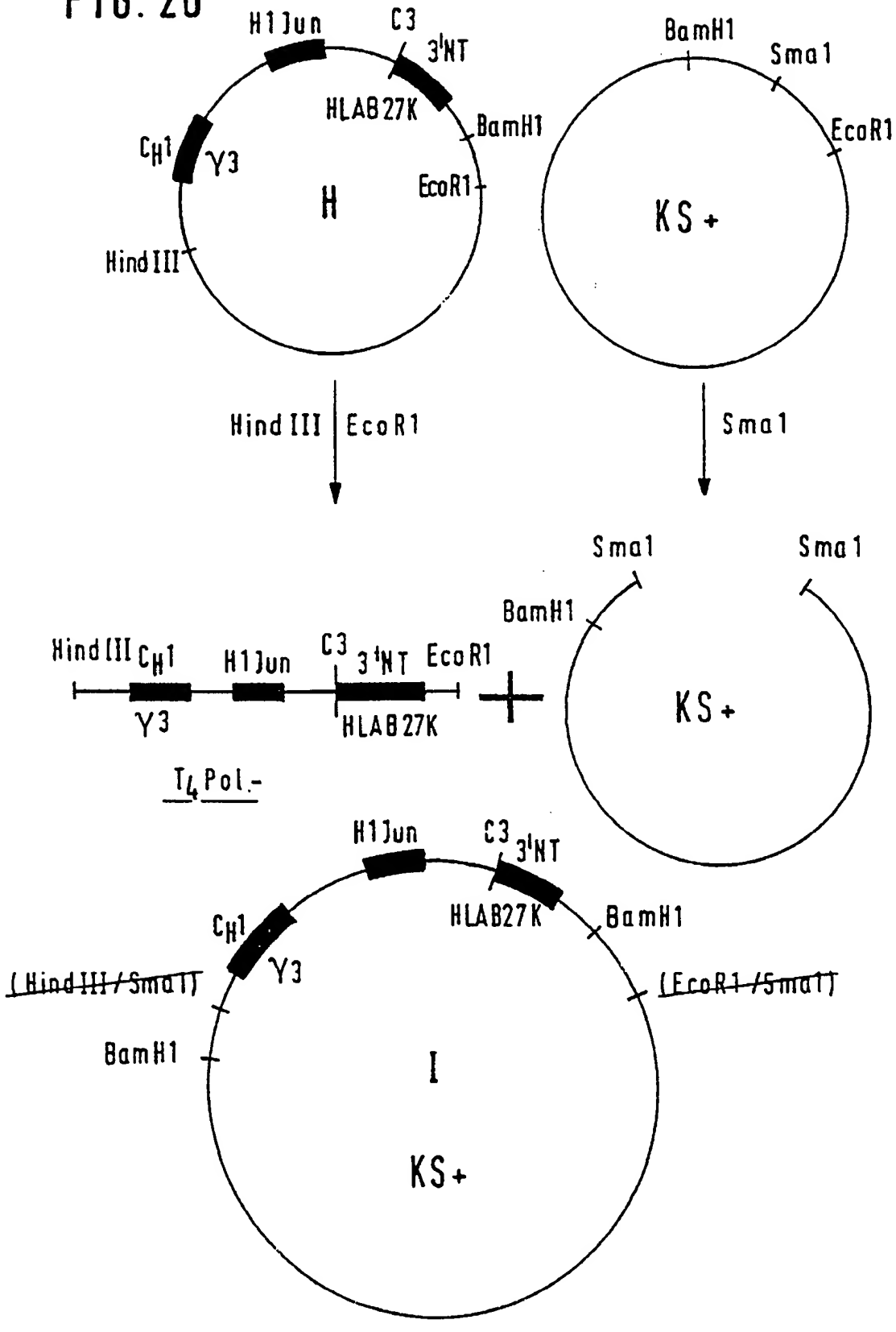
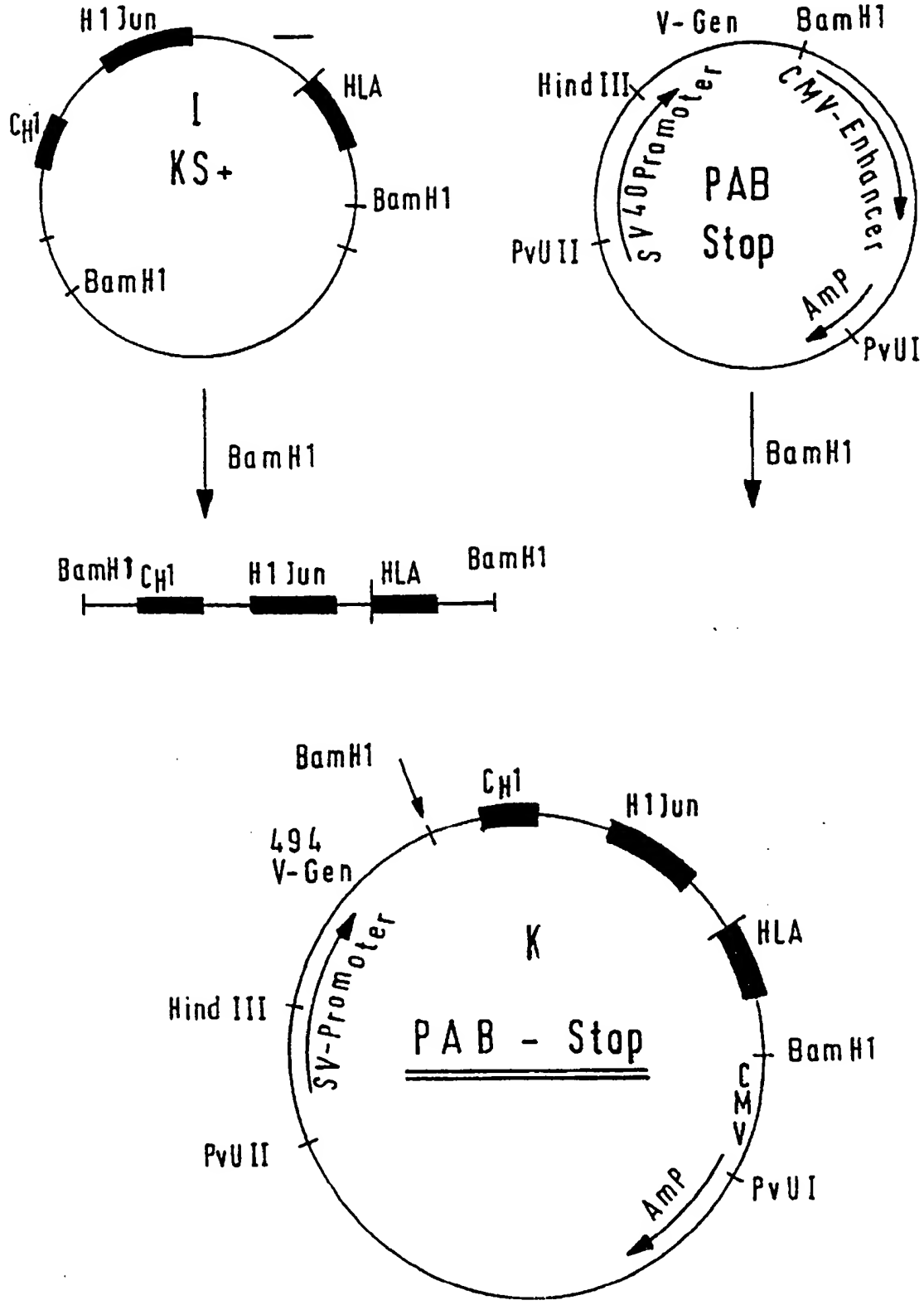

H1Jun
C3
3'NT
HLAB27K
BamH1
CH1
γ3
EcoR1
H
Hind III
BamH1
Sma1
EcoR1
KS+
Hind III
EcoR1
Sma1
Sma1
Sma1
BamH1
Hind III
CH1
H1Jun
C3
3'NT
EcoR1
γ3
HLAB27K
KS+
T4 Pol.-
H1Jun
C3
3'NT
CH1
HLAB27K
BamH1
γ3
(Hind III/Sma I)
(EcoR1/Sma I)
BamH1
I
KS+

FIG. 21


H1 Jun
HLA
CH1
I
KS+
BamH1
BamH1
V-Gen
BamH1
Hind III
SV40 Promoter
CMV-Enhancer
PAB
Stop
PvU II
AmP
PvUI
BamH1
BamH1
BamH1
CH1
H1 Jun
HLA
BamH1
BamH1
CH1
H1 Jun
494
V-Gen
SV-Promoter
K
HLA
Hind III
PAB - Stop
BamH1
CMV
PvU II
AmP
PvUI

VH

```
 Q V Q L Q E S G G G L V Q P G G S L R L
CAGGTCCAACTGCAGGAGTCTGGAGGAGGCTTGGTACAGCCTGGGGGTTCTCTGAGACTC
        10        20        30        40        50        60

 S C A T S G F S D Y Y M N W V R Q P P G
TCCTGCGCAACTTCTGGGTTCAGTGATTACTACATGAACTGGGTCCGCCAGCCTCCAGGA
        70        80        90       100       110       120

 K A L E W L G F I S N K P N G H T T E Y
AAAGCACTTGAGTGGTTGGGTTTTATTTCAAACAAACCTAATGGTCACACAACAGAGTAC
       130       140       150       160       170       180

 S A S V K G R F T I S R D N S Q S I L Y
AGTGCATCTGTGAAGGGTCGGTTCACCATCTCCAGAGATAATTCCCAAAGCATCCTCTAT
       190       200       210       220       230       240

 L Q M N T L R A E D S A T Y Y C A R D K
CTTCAAATGAACACCCTGAGAGCTGAGGACAGTGCCACTTATTATTGTGCAAGAGATAAG
       250       260       270       280       290       300

 G I R W Y F D V W G Q G T T V T V S S
GGAATACGATGGTACTTCGATGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
       310       320       330       340       350
```

VK

```
  A I L S A S P G E K V T M T C R A S S S
AGCAATCCTGTCTGCATCTCCAGGGGAGAAGGTCACAATGACTTGCAGGGCCAGCTCAAG
        10        20        30        40        50        60

  V S Y M H W Y Q Q K P G S S P K P W I Y
TGTAAGTTACATGCACTGGTACCAGCAGAAGCCAGGATCCTCCCCCAAACCCTGGATTTA
        70        80        90       100       110       120

  A T S N L A S G V P A R F S G S G S G T
TGCCACATCCAACCTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGGGAC
       130       140       150       160       170       180

  S Y S L T I I R V E A E D A A T Y Y C Q
CTCTTACTCTCTCACAATCATCAGAGTGGAGGCTGAAGATGCTGCCACTTATTACTGCCA
       190       200       210       220       230       240

  Q W S S N P L T F G A G T K L E I
GCAGTGGAGTAGTAACCCGCTCACGTTCGGTGCTGGGACCAAGCTGGAGATC
       250       260       270       280       290
```

FIG. 22

VH

```
 L  Q  E  S  G  P  D  L  V  K  P  S  Q  S  L  S  L  T  C  T
CTGCAGGAGTCAGGACCTGACCTGGTGAAACCTTCTCAGTCACTTTCACTCACCTGCACT
        10        20        30        40        50        60

 V  T  G  Y  S  I  T  S  G  Y  S  W  H  W  I  R  Q  F  P  G
GTCACTGGCTACTCCATCACCAGTGGTTATAGCTGGCACTGGATCCGGCAGTTTCCAGGA
        70        80        90       100       110       120

 N  K  L  E  W  M  G  Y  I  Q  Y  S  G  I  T  N  Y  N  P  S
AACAAACTGGAATGGATGGGCTACATACAGTACAGTGGTATCACTAACTACAACCCCTCT
       130       140       150       160       170       180

 L  K  S  R  I  S  I  T  R  D  T  S  K  N  Q  F  F  L  Q  L
CTCAAAAGTCGAATCTCTATCACTCGAGACACATCCAAGAACCAGTTCTTCCTGCAGTTG
190       200       210       220       230       240

 N  S  V  T  T  E  D  T  A  T  Y  Y  C  A  R  E  D  Y  D  Y
AATTCAGTGACTACTGAGGACACAGCCACATATTACTGTGCAAGAGAAGACTATGATTAC
       250       260       270       280       290       300

 H  W  Y  F  D  V  W  G  A  G  T  T  V  T  V  S  S
CACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCA
       310       320       330       340       350
```

VK

```
 L  T  Q  S  P  A  I  M  S  A  S  L  G  E  E  I  T  L  T  C
CTGACCCAGTCTCCAGCAATCATGTCTGCATCTCTAGGGGAGGAGATCACCCTAACCTGC
        10        20        30        40        50        60

 S  T  S  S  S  V  S  Y  M  H  W  Y  Q  Q  K  S  G  T  S  P
AGTACCAGCTCGAGTGTAAGTTACATGCACTGGTACCAGCAGAAGTCAGGCACTTCTCCC
        70        80        90       100       110       120

 K  L  L  I  Y  S  T  S  N  L  A  S  G  V  P  S  R  F  S  G
AAACTCTTGATTTATAGCACATCCAACCTGGCTTCTGGAGTCCCTTCTCGCTTCAGTGGC
       130       140       150       160       170       180

 S  G  S  G  T  F  Y  S  L  T  I  S  S  V  E  A  E  D  A  A
AGTGGGTCTGGGACCTTTTATTCTCTCACAATCAGCAGTGTGGAGGCTGAAGATGCTGCC
       190       200       210       220       230       240

 D  Y  Y  C  H  Q  W  S  S  Y  P  T  F  G  G  G  T  K  L  E
GATTATTACTGCCATCAGTGGAGTAGTTATCCCACGTTCGGAGGGGGGACCAAGCTGGAG
       250       260       270       280       290       300
```

FIG. 23

VH

```
 Q  V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V  K  M
CAGGTCCAACTGCAGCAGTCTGGACCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAGATG
        10         20         30         40         50         60

 S  C  K  A  S  G  Y  T  F  T  Y  Y  V  I  H  W  V  K  Q  K
TCCTGCAAGGCTTCTGGATACACATTCACTTACTATGTTATTCACTGGGTGAAACAGAAG
        70         80         90        100        110        120

 P  G  Q  G  L  E  W  I  G  Y  I  H  P  Y  N  A  G  T  E  Y
CCTGGGCAGGGCCTTGAGTGGATTGGATACATTCATCCTTACAATGCTGGTACTGAGTAC
       130        140        150        160        170        180

 N  E  K  F  K  G  K  A  T  L  T  S  D  K  S  S  S  T  A  Y
AATGAGAAGTTCAAAGGCAAGGCCACACTGACTTCAGACAAATCCTCCAGCACAGCCTAC
       190        200        210        220        230        240

 M  E  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  S  M  G  R
ATGGAGCTCAGCAGCCTGACCTCTGAGGACTCTGCGGTCTATTACTGTTCAATGGGACGA
       250        260        270        280        290        300

 G  G  D  Y  W  G  Q  G  T  T  V  T  V  S  S
GGGGGTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
       310        320        330        340
```

VK

```
 L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K  V  T  M  T  C
CTGACCCAGTCTCCAGCAATTATGTCTGCATCTCCTGGGGAGAAGGTCACCATGACCTGC
        10         20         30         40         50         60

 S  A  S  S  S  V  S  Y  M  H  W  Y  Q  Q  K  S  G  T  S  P
AGTGCCAGCTCAAGTGTAAGTTACATGCACTGGTACCAGCAGAAGTCAGGCACCTCCCCC
        70         80         90        100        110        120

 K  R  W  I  Y  D  T  S  K  L  A  S  G  V  P  A  R  F  S  G
AAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGC
       130        140        150        160        170        180

 S  G  S  G  T  S  Y  S  L  T  I  S  S  M  E  A  E  D  A  A
AGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGCATGGAGGCTGAAGATGCTGCC
       190        200        210        220        230        240

 T  Y  Y  C  Q  Q  W  S  S  N  P  F  T  F  G  A  G  T  K  L
ACTTATTACTGCCAGCAGTGGAGTAGTAACCCATTCACGTTCGGCGCGGGGACCAAGCTG
       250        260        270        280        290        300
```

FIG. 24

VH

```
 A  E  S  G  P  G  L  V  R  L  T  S  L  S  I  T  C  T  V  S
GCAGAGTCAGGGCCTGGCCTGGTGCGCCTCACGAGCCTGTCCATCACTTGCACTGTCTCT
        10        20        30        40        50        60

 G  F  S  L  I  S  Y  G  V  H  W  V  R  Q  P  P  G  K  G  L
GGCTTTTCATTAATTAGTTATGGTGTACACTGGGTTCGCCAGCCTCCAGGAAAGGGTCTG
        70        80        90       100       110       120

 E  W  L  G  V  I  W  A  G  G  S  T  N  Y  N  S  A  L  M  S
GAGTGGCTGGGAGTAATATGGGCAGGTGGAAGCACAAATTATAATTCGGCTCTCATGTCC
       130       140       150       160       170       180

 R  L  S  I  S  K  D  N  S  K  S  Q  V  F  L  K  M  N  S  L
AGACTGAGCATCAGCAAAGACAACTCCAAGAGCCAAGTTTTCTTAAAAATGAACAGTCTG
       190       200       210       220       230       240

 Q  T  G  D  T  A  I  Y  Y  C  A  R  G  G  D  D  Y  D  G  F
CAAACTGGTGACACAGCCATATACTACTGTGCCAGAGGGGGGGATGATTACGATGGGTTT
       250       260       270       280       290       300

 A  Y  W  G  Q  G  T  T  V  T  V  S  S  G  E  S
GCTTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAGGTGAGTCC
       310       320       330       340
```

VK

```
 L  T  Q  S  P  S  S  L  A  V  S  A  G  E  K  V  T  M  S  C
CTGACCCAGTCTCCATCCTCCCTGGCTGTGTCAGCAGGAGAGAAGGTCACTATGAGCTGC
        10        20        30        40        50        60

 K  S  S  Q  S  L  L  S  S  T  K  R  K  N  Y  L  A  W  Y  Q
AAATCCAGTCAGAGTCTGCTCAGCAGTACAAAGCGAAAGAACTACTTGGCTTGGTACCAG
        70        80        90       100       110       120

 Q  K  P  G  Q  S  P  K  L  L  I  Y  W  A  S  T  R  E  S  G
CAGAAACCAGGTCAGTCTCCTAAACTACTGATCTACTGGGCATCCACTCGGGAATCTGGG
       130       140       150       160       170       180

  Y  P  D  R  F  T  G  S  G  S  G  T  D  F  T  L  T  I  S  S
GTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGT
       190       200       210       220       230       240

 V  Q  A  E  D  L  A  V  Y  Y  C  K  Q  S  Y  N  L  R  A  F
GTGCAGGCTGAAGACCTGGCAGTTTATTACTGCAAACAATCTTATAATCTTCGGGCGTTC
       230       260       270       280       290       300

 G  G  G  T  K  L  E  I  K
GGTGGAGGGACCAAGCTGGAGATCAAA
       310       320
```

FIG. 25

BISPECIFIC AND OLIGOSPECIFIC MONO-AND OLIGOVALENT RECEPTORS, THE PREPARATION AND USE THEREOF

This application is a continuation, of application Ser. No. 08/147,428, filed Nov. 5, 1993, now abandoned, which is a continuation of application Ser. No. 8/017,439 filed Feb. 12, 1993, abandoned; which is a continuation of application Ser. No. 07/541,020 filed Jun. 20, 1990, now abandoned.

The invention relates to bispecific and oligospecific, mono- and oligovalent receptors which are prepared by gene manipulation by fusion of DNA coding for F(ab) fragments of antibodies of two or more different specificities by means of suitable linkers. In this connection, one specificity is preferably directed either against an epitope of a tumor-associated antigen (TAA), which is located on the cell membrane or in the interstitium or against an epitope in the tumor endothelium (TE), while the other specificities relate to high-molecular or low-molecular weight ligands and react, for example, with the chelates ethylenediaminetetraacetate and diethylenetriaminepentaacetate in Y90 complexed form (EDTA-Y90 and DTPA-Y90 respectively). In a particularly preferred embodiment, the binding with the chelates takes place on the chelate receptor arm via fos-jun interaction (or else avidin-biotin interaction). Other preferred specificities have catalytic properties, or bind to other TAA's on the same tumour cell or to receptors on lymphoid cells.

Bispecific antibodies have to date been prepared by the following methods

- chemical coupling of antibodies of diverse specificity via heterobifunctional linkers (H. Paulus, Behring Inst. Mitt. 78, (1985), 118–132)
- fusion of hybrids which are already available and secrete various monoclonal antibodies (NAb), and isolation of the bispecific monovalent portion (U. S. Staerz and M. J. Bevan, Proc. Natl. Acad. Sci. U.S.A. 83, (1986) 1453–1457)
- transfection of the light and heavy chain genes of two different Mabs (4 genes) into murine myeloma cells or other eukaryotic expression systems and isolation of the bispecific monovalent portion (U. Zimmermann, Rev. Physio. Biochem. Pharmacol. 105 (1986), 176–260; J. van Dijk et al., Int. J. Cancer 43, (1989), 344–349).

Bispecific antibodies of this type are employed for the therapy and diagnosis of malignant tumors. The principle of the process comprises, in the first step, achieving saturation of the epitopes which are recognized by one of the two specificities on the target cells, by injection of the bispecific macromolecule over prolonged periods and with high doses.

In the second step, which comprises interruption of the treatment for several days, there is autoelimination of the non-specifically adsorbed bispecific antibody from the non-target tissues.

This autoelimination can be speeded up by injection of an anti-idiotype antibody which is coupled to sugar residues, preferably galactose, and is directed against the anti-tumor arm of the bispecific receptor.

The third step in the process comprises i.v. injection of a radiolabeled, hydrophilic low-molecular weight ligand which does not accumulate in cells, has a short residence time in the body, has high complexing constants for beta- and gamma-emitters such as ^{90}Y, ^{186}Re, ^{188}Re, ^{189}Re, ^{99m}Tc or ^{111}, and binds to the second specificity of the bispecific receptor with high affinity. This step results in a concentration of the radioactive ligand, associated with prolonged

retention on the target tissue, which results in selective destruction of the target tissue and makes possible diagnosis of metastases, for example.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts the gene fragments which code for the V_H and C_H1 sections of monoclonal antibodies a and b being linked in such a manner that the N-terminus of the V_H domain of Mab b is covalently bonded via a polypeptide spacer to the C terminus of the C_H1 domain of MAb a.

FIG. **2** depicts the preferential expression product when the gene construct of FIG. **1** is transfected together with the genes for the light chains of MAb a and MAb b into eukaryotic cells. The correct pairing of heavy and light chains is aided by modification of the C_H1a, C_H1b, C_ka and C_kb domains such that opposite charges meet at the constant domains or areas of contact which are each hydrophobic or each hydrophilic.

FIG. **3** depicts bispecific receptors that are bivalent for MAb a and monovalent for MAb b. The C-terminal end of the C_H1 domain of MAb b of the gene construct depicted in FIG. **1** is linked to the N-terminal end of the V_H domain of MAb a by a polypeptide spacer.

FIG. **4** depicts the preferential expression product when the gene construct depicted in FIG. **3** is transfected together with the genes for the light chains of MAb a and MAb b into eukaryotic cells. The fusion molecules expressed preferentially comprise two F(ab) fragments of MAb a and one F(ab) fragment of MAb b.

FIG. **5** depicts a plasmid containing the C_H1 exon and a hinge exon.

FIG. **6** depicts plasmid M containing the C_H1 exon and the 3' NT region of an HLA B27 gene.

FIG. **7** depicts an M13 vector containing the V_Ha and V_Hb genes.

FIG. **8** depicts the plasmid pEV_H a C containing the V_Ha gene.

FIG. **9** depicts the construction of plasmid clone L.

FIG. **10** depicts the construction of plasmid X.

FIG. **11** depicts the construction of plasmid Z.

FIG. **12** depicts the construction of plasmid Y.

FIG. **13** depicts the construction of plasmid PEVT.

FIG. **14** depicts plasmid D containing the CH1 exon and the first hinge oxon of the IgG3 C gone.

FIG. **15** depicts plasmid E containing the C3 exon and the 3' NT region of the HLA B27k gene.

FIG. **16** depicts the construction of plasmid F.

FIG. **17** depicts the construction of plasmid G.

FIG. **18** depicts plasmid G' containing a SstI and a SphI restriction cleavage site at the 3'-end of the hinge exon and without a SstI restriction cleavage site at the 5' end of the hinge exon.

FIG. **19** depicts the construction of plasmid H.

FIG. **20** depicts the construction of plasmid I.

FIG. **21** depicts the construction of plasmid K.

FIG. **22** depicts the DNA and amino acid sequence of V_H and V_K for MAb A.

FIG. **23** depicts the DNA and amino acid sequence of V_H and V_K for MAb B.

FIG. **24** depicts the DNA and amino acid sequence of V_H and V_K for MAb C.

FIG. 25 depicts the DNA and amino acid sequence of V_H and V_K for MAb D.

The invention now provides bispecific and oligospecific receptors which have, depending on requirements, mono- or oligovalent binding sites to the particular epitopes and are produced by gene manipulation by means of suitable linkers. This entails the gene fragments which code for the V_H and C_H1 sections of antibodies a and b being linked, by means of suitable synthetic oligonucleotides as depicted by way of example in Tab. 1, in such a manner that the N-terminus of the V_H domain of MAb b is covalently bonded via a polypeptide spacer to the C terminus of the C_H1 domain of MAb a (FIG. 1). The $V_Ha C_Ha$-polypeptide spacer $-V_Hb C_H1b$ gene construct is transfected together with the genes for the light chains of antibodies a and b into eukaryotic cells (for example mouse myeloma cells). The C_H1a, C_H1b, C_ka and C_kb domains are modified in such a way that opposite charges meet at the areas of contact of the constant domains ($C_H1a(+)C_ka(-)$; $C_H1b(-)C_kb(+)$) (+=positive, −=negative) or opposing areas of contact are in each case hydrophobic or in each case hydrophilic. This means that the transfectomas preferentially express hybrid molecules which have the correct pairings of heavy and light chains (FIG. 2).

Here, antibody a is a representative example of an antitumor antibody, and antibody b represents an antibody against a low-molecular weight ligand, preferably the chelates DTPA-Y90 or EDTA-Y90.

Bi- or oligospecific receptor accordingly means a genetically engineered construction of V_H and C_H1 domains of antibodies of diverse specificity via suitable linkers so that the required mobility for association with the corresponding light chains is present and antigen binding is not impeded.

The antigen-binding sites are called valencies or binding sites. A bispecific monovalent receptor thus has one antigen-binding site in each case when there are two specificities. Consequently, a bispecific trivalent receptor has one antigen-binding site for one specificity and two antigen-binding sites for the other.

Bispecific receptors which are bivalent for the tumor antigen (MAb a) and monovalent for EDTA-Y90 (MAb b) are prepared by linking the heavy chain gene construct described above, by means of the abovementioned oligonucleotide linker, to the gene section which codes for the V_H and C_H1 domains of MAb a (FIG. 3) so that the C-terminal end of the C_H1 domain of MAb b is connected to the N-terminal end of the V_H domain of MAb a by a polypeptide spacer. These gene constructs are transfected together with the genes for the light chains belonging to MAb a and b into eukaryotic cells (for example myeloma cells). The C_H1 and C_k domains are provided, as described above, with complementary charges or areas of contact which are in each case hydrophobic or hydrophilic. The transfectomas preferentially express fusion molecules which comprise two F(ab) fragments of MAb a and one F(ab) fragment of MAb b (FIG. 4). The mobility of the peptide linkers makes possible the alignment of the two F(ab) arms of MAb a towards the tumor cell with, at the same time, alignment of the F(ab) arm of MAb b towards the intercellular space. It is possible correspondingly to add further binding sites of identical or different specificity. Moreover, it is possible to combine freely the sequence of specificities in the constructs.

Consequently, the invention relates to bispecific or oligospecific, mono- or oligovalent receptors which have both specificity for an epitope located on the cell membrane or in the interstitium, for example TAA or TE, and specificity for a low- or high-molecular weight ligand which is distributed exclusively in the extracellular space. In this connection, one specificity is preferably formed by the tumor-specific antibodies, as proposed in German Patent Application P 39 09 799.4, whereas the other specificity is preferably directed against DTPA-Y90 or EDTA-Y90. In a particularly preferred embodiment, the binding takes place with chelates on the chelate receptor arm via fos-Jun interaction (see Example 5). Another preferred variant of the invention comprises the incorporation of specificities with catalytic activity. Moreover, there is no restriction on the choice of the sequence of the specificities or binding valencies, as is shown by way of example in FIG. 4 for three valencies with two specificities.

Particularly preferred constructs according to the invention are those which contain a V gene of Tables 2, 3, 4 and/or 5. Antibodies with these sequences and their properties are described in German Patent Application P 39 09 799.4. Moreover, the complementarity determining regions (CDRs) can be identified by the method of Kabat and Wu (Sequences of Proteins of Immunological Interest, U.S. Dept. of Health and Human Services, U.S. Government Printing Office (1987)). Likewise preferred are constructs which contain specificities against the epitopes defined by the monoclonal antibodies described above.

The invention additionally relates to genetic engineering processes for the preparation of the constructs described above, and to a use of the abovementioned constructs for preparing pharmaceuticals for controlling and diagnosing target cells. This entails, in a first step, saturation of the relevant epitopes on target cells after injection of the constructs and, in a subsequent interval, elimination of non-specifically adsorbed or unbound constructs. The step following this comprises injection and subsequent specific binding of a low- or high-molecular weight ligand which does not accumulate in cells and is intrinsically cytotoxic or is "activated" to cytotoxicity by extracorporeal influences where appropriate in another step. Examples of processes of this type are enzymatic activation, activation by microwave irradiation of a prodrug or activation by laser light.

The invention is furthermore contained in the examples and the patent claims.

EXAMPLE 1

Preparation of an anti-DTPA-Y90 or EDTA-Y90 MAb

Isothiocyanatobenzyl-DTPA (formula 2) was covalently coupled as hapten onto human serum albumin (HSA as carrier) with a degree of derivatization of 19 benzyl-DTPA molecules per HSA molecule by the method described in (N. W. Brechbiel et al., Inorganic Chemistry25, (1986) 2772–2781). 20 μg of this hapten-carrier complex, into which cold Y had been complexed, were injected s.c. on day 0 with Freund's adjuvant, on day 7 and 14 with incomplete Freund's adjuvant and on day 21 with PBS into Balb/c mice. On day 24, the spleens of the mice with the highest anti-DTPA antibody titers were fused with the SP2/0-Ag14 myeloma cell line (Shulman et al., Nature 276, (1978) 269). The resulting hybridomas were tested in a DTPA-specific ELISA for the production of high-affinity MAbs against DTPA and EDTA. The ELISA comprised a solid phase which was loaded with a solution containing HSA-benzyl-DTPA-Y. The supernatant containing the MAb to be tested was preincubated with free chelate or its metal ion complexes, and its binding to the specific solid phase was

measured. An enzyme amplification system which is coupled to an anti-mouse immunoglobulin antibody was used for this purpose. The details of these methods are described in Annex 1a and 1b.

MAbs which have the properties described in Annex 1e were obtained using this assay system.

In contrast to many other anti-DTPA/EDTAMAbs, these MAbs do not bind to normal human tissue, as was found using the APAAP technique (Cordell et al., J. Histochem. Cytochem. 32: 219, 1984) on cryopreserved tissues. It is therefore possible to use these MAbs in vivo for diagnosis and therapy.

The competitors employed were the chelates DTPA and EDTA in non-complexed and in complexed form (Annex 1c).

In addition, the structurally related compounds transaconitic acid and 1,2-diaminoethane were used as inhibitors (see Annex 1e). MAb BW 2050/174 is particularly suitable for in vivo use, showing preferential binding to EDTA-Y, in contrast to all the other MAbs (see Annex 1e, low competitor excess for EDTA-Y (100×) higher excess for other EDTA Komplexons). The hybrid 2050/174 was therefore stabilized and used for developing the EDTA-Y arm in the bispecific receptor.

EXAMPLE 2

Preparation and expression of a V_H1a C_H1-linker-V_H1b C_H1 gene construct

The techniques used here were taken, unless indicated otherwise, from Molecular Cloning, A Laboratory Manual; Sambrook, Fritsch, Maniatis; Cold Spring Harbor Laboratory, 1982 (pp. 11–44, 51–127, 133–134, 141, 146, 150–167, 170, 188–193, 197–199, 248–255, 270–294, 310–328, 364–401, 437–506) and from Molecular Cloning, A Laboratory Manual, Second Edition; Sambrook, Fritsch, Maniatis; Cold Spring Harbor Laboratory Press, 1989, (pp. 16.2–16.22, 16.30–16.40, 16.54–16.55).

A human IgG_3 C gene was isolated from a human gene bank in EMBL3 phages (A. M. Frischauf et al., J. Mol. Biol. 170, 827–842 (1983) and G. H. A. Seemann et al., The EMBO Journal 5 (1986), 547–552).

Constructions which contain, on the one hand, only the C_H1 exon and a hinge exon (FIG. 5) and, on the other hand, the C_H1 exon and the 3'NT region of an HLAB27 gene (FIG. 6, fragment M in plasmid M) were prepared from this IgG_3 C gene as described in German Patent Application P 38 25 615.0.

The V_Ha and V_Hb genes were amplified from mRNA of the hybrid clones a and b as described by Orlandi et al. (Proc. Natl. Acad. Sci. U.S.A. 86, (1989), 3833–3837) and cloned in an M13 vector (V_Ha PCR and V_Hb PCR) (FIG. 7). The V_Ha gene was cloned as the HindIII-BamHI fragment into the eukaryotic expression vector pEV_H (Simon et al., Nucl. Acids. Res. 16, (1988), 354) (FIG. 8). The result is the plasmid pEV_H a C.

The human IgG C gene subclone with the C_H1 and with one hinge exon (FIG. 5) contains a PstI cleavage site between C_H1 exon and hinge exon. The V_H genes contain a PstI cleavage site at the 5' end. The linker oligonucleotide is designed such that it overlaps at the 5' end with the region of the PstI cleavage site on the C_H1+1H subfragment of the IgG C gene and at the 3' end with the PstI cleavage site of the V_Hb gene. The linker oligonucleotide is cloned by means of its PstI cleavage sites into the PstI cleavage site of a PUC 18 plasmid (FIG. 9). The result is the plasmid clone L.

The plasmid with the IgG_3 C gene subfragment with C_H1 exon and with a hinge exon is cleaved with PstI and BamHI and ligated to the V_Hb gene fragment cut out of V_Hb PCR as PstI-BamHI fragment (FIG. 10). The result is the plasmid X.

The plasmid X is cleaved with PstI and ligated to the linker fragment which has been cut out of the plasmid L likewise with PstI (FIG. 11). Nucleic acid sequence analysis is used to identify the clone Z in which the linker is cloned in correct orientation between C_H1 and V_Hb without disturbing the intron/exon junction between intron 3 and linker exon and without disturbing the reading frame at the junction between linker and V_Hb gene.

The plasmid pEVa C is cleaved with BamHI and ligated to the fragment M cut out of the plasmid M with BamHI. Restriction analysis is used to identify the clone Y which contains the fragment M in the correct orientation (FIG. 12).

The plasmid Y is partially cleaved with BamHI and ligated to the fragment (C_H1-linker-V_Hb) cut out of the plasmid X by HindIII and BamHI cleavage, after all the ends have been filled in. Nucleotide sequence analysis and restriction mapping are used to identify the plasmid clone PEVT which contains the fusion geneV_Ha C_H1-linker-V_Hb C_H1 with the correct orientation of all the exons (FIG. 13).

The plasmid PEVT is transfected together with plasmids which harbor the genes for the light chains of antibodies a and b into suitable eukaryotic expression cells in order to express the antibody a F (ab) antibody b F (ab) fusion protein.

EXAMPLE 3

Transfection of the light and heavy chain genes of two different MAbs (4 genes)

The isolation of immunoglobulin genes is described in German Patent Application P 39 09 799.4.

The genes cloned into vectors were transfected by electroporation after linearization of the vectors into X63Ag8.653 myeloma cells (H. Stopper et al., Biochem. Biophys. Acta 900 (1987), 38–44). The transfectomas which grew in selective media were tested for the production of bispecific monovalent MAbs in a specific RIA. This RIA comprised TAA adsorbed on a solid phase, onto which, after blockade of the non-specific sites by casein, the transfectoma supernatants to be assayed were placed. After DTPA or EDTA which were complexed with ^{90}Y or ^{99m}Tc had been added and the excess had been washed away, it was possible to detect those transfectomas which secreted bispecific monovalent anti-TAAxanti-EDTA MAbs by an increased radioactive signal on the solid phase.

Transfectoma 9 was stabilized by limited dilution cloning and expanded in cell culture. Cell culture supernatants were concentrated 10×, the MAb fraction was purified by protein A chromatography (P. L. Ey et al., Immunochemistry 15, (1978), 429), and the fraction containing the bispecific monovalent MAb was purified by anion exchange chromatography (J. Van Dijk et al., Int. J. Cancer 43, (1989), 344–349).

EXAMPLE 4

Biological effectivity

Purified protein containing the bispecific monovalent MAb (BW 431/26×BW 2050/174) was injected i.v. in 500

μg doses on days 0, 3, 5, 8, 10 and 12 into nude mice carrying human tumor xenografts (CoCa 4). 50 μCi of EDTA-Y90 were injected i.v. into each of the animals of day 27–30. A 2nd group of animals received on the same days 500 μg of MAb BW 431/26 in place of the bispecific MAb, and the EDTA-Y90 injections as described above.

A third tumor-carrying group received injections of PBS (as tumor-growth control) in place of the MAb and EDTA-Y90. Tumor growth was followed for 6 weeks. Injection of EDTA-Y90 resulted in significant inhibition of tumor growth in the group which received the bispecific monovalent MAb, whereas the animals injected with MAb BW 431/26 and treated with EDTA-Y90 showed no inhibition of tumor growth, compared with animals which received only PBS.

These data indicate the selective tumor-therapeutic efficacy of the bispecific monovalent MAb in combination with EDTA-Y90 as toxic principle.

Even more favorable tumor-therapeutic effects are obtained by the oligovalent/bispecific or oligospecific receptors because they remain longer on the tumor, because of the bivalent binding to TAA, and thus the ligand is likewise retained on the tumor for longer and in higher concentrations.

EXAMPLE 5

Optimization of the biological effectivity of bi- or oligospecific macromolecules by increasing the avidity of the anti-chelate arm

A crucial factor which influences the efficient attachment of the hydrophilic chelate undergoing extracellular distribution to the anti-chelate arm of the oligospecific macromolecule is the avidity of this arm for the chelate. The avidities of monoclonal antibodies for their corresponding epitopes are in the range 10^5–10^{11}/mol. Since these binding strengths are possibly insufficient to localize on the tumor the mass of chelate necessary for efficient radioimmunotherapy, in the following example the extremely strong interaction of the fos-leucine-zipper peptide (fos-peptide) with the jun-leucine-zipper peptide (jun-peptide) (Erin K. O'Shera et al., Science, 245, 1989) was used to immobilize the chelate as firmly as possible on the anti-chelate arm. It is necessary, in order to be able to utilize this strong fos-jun interaction, preferably to link the fos-peptide covalently to the chelate (DTPA). It is possible for this purpose to react in a first step isothiocyanatobenzyl-DTPA with hydrazine (or a diaminoalkane). The DTPA-benzylthiocarbazide produced in this way can be reacted, in a 2nd step, with N-(gamma-maleimidobutyryloxy)succinimide or an analog to give DTPA-benzyl-(gamma-maleimidobutyryl)thiocarbazide. Then, in a 3rd step, this compound is linked to the fos-peptide which has been extended by glycine-glycine-cysteine (FIG. 1) via the free SH group of the amino-terminal cysteine. The fos-peptide-DTPA conjugate produced in this way is complexed in a 4th step with yttrium chloride. The fos-peptide-DTPA-Y conjugate complex produced in this way can then be used for in vivo addition onto the jun-peptide arm of the bi- or oligospecific macromolecule. The synthesis of the example outlined above is described in detail hereinafter:

A) Preparation of the fos-EDTA-Y conjugate complex

Step 1:

Synthesis of EDTA-benzylthiocarbazide

Isothiocyanatobenzyl-EDTA (SCN-Bn-EDTA) (30 mg, 54 μmol) was stirred in 10% (v/v) aqueous hydrazine for 1 h. The solvent was then removed under high vacuum, and the residue was dried over phosphorus pentoxide under high vacuum and finally freeze-dried. The product was neutralized with DOWEX WX 2 (H^+ form) and again freeze-dried (yield 28 mg).

Step 2:

Synthesis of EDTA-benzyl(gamma-maleimidobutyryl)thiocarbazide

The EDTA-benzylthiocarbazide prepared in step 1 (20 mg; 34 μmol) and N-(gamma-maleimidobutyryloxy)succinimide (8 mg, 29 μmol=0.9 equiv.) were stirred in anhydrous dimethylformamide for 1 h. The mixture was then evaporated to dryness, and the residue was dried under high vacuum.

Step 3:

Coupling of the EDTA-benzyl(gamma-maleimidobutyryl)thiocarbazide to the amino-terminal cysteine in the fos-peptide

A solution of the fos-peptide (4.8 mg, 1 μmol) (see step 3.1) in phosphate-buffered saline (2 ml) was mixed with a suspension of the product mixture obtained as in step 2 (4 mg) in dimethylformamide (400 μl) and incubated at room temperature for 1 h. The reaction mixture was then subjected to gel filtration on a Sephadex G15 column in phosphate-buffered saline. The protein-containing eluate was collected and preserved at –30° C. (yield 4.2 mg).

Step 3.1:

Amino acid sequence of the fos-peptide (I) with N-terminal GGC extension.

Ac-CGGyLTDTLQAETDQLEDKKSALQTE-IANLLKEKEKLEFILAAYy The letters represent the following amino acids: A=alanine, C=cysteine, D=aspartic acid, E=glutamic acid, G=glycine, I=isoleucine, K=lysine, L=leucine, M=methionine, N=asparagine, Q=glutamine, R=arginine, S=serine, T=threonine, V=valine, Y=tyrosine.

The oligopeptide was synthesized using an automatic peptide synthesizer (Applied Biosystems Model 430A) by the Merrifield solid-phase method (Stewart and Young, Solid Phase Synthesis, Pierce Chemical Company, 2nd edition, Rockford Ill.) with the tert-butyloxycarbonyl protective group. The oligopeptides were cleaved off the phenylacetamidomethyl-polystyrene support. After elimination of the protective groups (Tom et al., 1983, J. Am. Chem. Soc. 105, 6442–6455) the oligopeptides were purified by reversed phase chromatography (PepRPC column, Pharmacia) as described by Rivier et al. (J. Chromatography 288, 303–328, 1984).

Step 4:

Preparation of a fos-peptide-EDTA-yttrium chelate with a fos-peptide-EDTA conjugate prepared as in step 3

The fos-peptide-EDTA conjugate prepared as in step 3 (4.2 mg) was dialysed against isotonic saline/0.1M sodium citrate, pH 7.0, in a dialysis tube with an exclusion limit of m.w. 1,000 (Spectrum), and was mixed with 6 mg of yttrium chloride which were dissolved in 3 ml of isotonic saline/0.1M sodium citrate, pH 7.0. After 1 h, back-dialysis against phosphate-buffered saline was carried out, and the chelate solution was preserved at –30° C. The fos-peptide-EDTA-Yttrium chelate described in the above example is then used as ligand in order to bind with high avidity to the jun-peptide arm of the bi- or oligospecific macromolecule. The construction of a bispecific macromolecule particularly suitable for this interaction is described in the following example.

B) Construction of the MAb-jun fusion molecule

The techniques used here were taken, unless otherwise indicated, from Maniatis et al. (Laboratory Manual EMBL

(1982), Heidelberg), and Sambrook (Molecular Cloning: A Laboratory Manual).

Step 1:

A human IgG3 C gene was isolated from a human gene bank in EMBL 3 phages (A. M. Frischauf et al., J. Mol. Biol. 170, 827–842, 1983 and G. H. A. Seemann et al., The EMBO Journal 5, 547–552, 1986). A construction (D) which contains only the CH1 exon and the first hinge exon of the IgG3 C gene (FIG. **14**) was prepared from this IgG3 C gene as described in German Patent Application P 3825615.0.

A human HLA B27k gene was isolated from the same gene bank as likewise described in German Patent Application P 3825615.0. A construct (E) which contains only the C3 exon and the 3' NT region of the HLAB27k gene (FIG. **15**) was prepared from this HLAB27k gene.

Step 2:

The C1 exon and the 3' NT region of the HLAB27k gene were cut out of the plasmid E with XbaI, and the fragment was isolated and cloned into the XbaI cleavage site of the construct D. Restriction analysis and nucleic acid sequence analysis were used to identify the clone F which contains the C3 exon and the 3' NT region of the BLAB27k gene in the correct 5'-3' orientation 3' from the IgG3 C gene fragment (FIG. **16**).

Step 3:

The insert of the clone F is cut out of the plasmid using the endonucleases HindIII and EcoRI and cloned between the HindIII and EcoRI cleavage sites of an M13mp18 double-stranded (DS) phage. The phage clone G which contains the antibody/HLA fusion gene fragment is isolated (FIG. **17**).

Step 4:

Uracil single strands are prepared from the phage clone G by the method of T. A. Kunkel, 1985, Proc. Natl. Acad. Science, U.S.A., 82, 488–492. The single-stranded phages were hybridized with the mutagenic oligonucleotides 1 and 2 (Tab. 6), and the gaps between the oligonucleotides were closed with Klenow DNA polymerase and T4 ligase. After transformation into *E. coli*, restriction analysis and nucleic acid sequence analysis were used to identify a phage clone (G) in which the SstI restriction cleavage site at the 5' end of the hinge exon had been deleted. At the same time, a SstI and a SphI restriction cleavage site were introduced at the 3' end of the hinge exon (FIG. **18**). To delete the SstI cleavage site, the third base of the 2nd codon of the hinge exon was converted from C into G, and to introduce the SstI and SphI cleavage sites, the bases 5'GAGCTCGGGGCA3' were introduced between the 15th and 16th codon of the hinge exon (Tab. 7).

Step 5:

Double-stranded DNA of the phage clone G' is cleaved completely with SphI and partially with SstI. The synthetic oligonucleotides Jun I and Jun II (Tab. 8) are combined to give a double-stranded DNA fragment which contains at each of its ends a cut SphI and SstI restriction cleavage site and codes for a peptide which contains the Jun leucine zipper (O'Shea et al., Science, 245, 646–648, 1989).

The double-stranded DNA fragment is cloned into the SstI and SphI restriction cleavage sites of the F' phage clone, and the phage clone H which contains a gene construct in which the sequence for the Jun zipper peptide is inserted in the hinge exon is identified (FIG. **19**).

Step 6:

The insert of the DS phage H was cut out with the restriction endonucleases HindIII and EcoRI, the ends were filled in with T4 polymerase and cloned into an SmaI-cleaved KsF vector (Stratagene, 11099 North Torrey Pines Road, La Jolla Calif. 92037). Plasmid clone I which contains the antibody/Jun/HLA fusion gene in the orientation (FIG. **20**) in which it is flanked on both sides by a BamHI cleavage site was identified.

Step 7:

The antibody/Jun/HLA fusion gene was cut out of the KS clone I with BamHI and cloned into the expression plasmid pABStop (Behringwerke AG) which contains a specific functional immunoglobulin V gene. The specific V gene was obtained as described in Patent Application P 3909799.4. The expression plasmid K which contains the antibody/Jun/HLA fusion gene construct in the correct orientation downstream of the V_H gene was identified (FIG. **21**).

Cotransformation of the plasmid K with a plasmid which contains the gene for the light chain of the specific MAb, and a plasmid which carries a resistance gene, leads to expression of a specific antibody $F(ab')_2$ fragment which contains in the hinge region two Jun zipper peptides, with the Jun zipper peptide being modified in such a way that there is no longer any homodimer (Jun/Jun) formation.

EXAMPLE 6

Optimization of the amount of bi- or oligospecific receptor on the tumor and minimization thereof in the blood and normal tissues

Scientific investigations by others have shown that penetration of solid tumors by macromolecules >50 kDa takes place slowly, and usually only the edge region or a few areas in the tumor are reached. These investigations are based on experiments which comprise a single injection of small amounts of macromolecules. In contrast thereto, we have found that substantial penetration of the entire tumor mass in nude mouse xenografts is possible by a repetitive i.v. injection of large amounts of bi- or oligospecific receptors (10×250 µg receptor/mouse for 10 days). Furthermore, because of their specific binding to TAA, the bi- or oligospecific receptors remain attached for long periods (>20 days) in large amounts on the tumor cell membrane and in the tumor interstitium. These results were obtained using the indirect alkaline phosphatase technique on cryopreserved thin sections of human colonic and pancreatic tumor xenografts.

During this time (after only 10 days) the bi- or oligospecific receptor molecules had already been eliminated from the TAA-negative normal tissues and the blood by degradation and excretion. In order to shorten this elimination period, an anti-idiotype MAb (anti Id) which reacts only with the anti-TAA arm of unbound bi- or oligospecific receptor molecules was injected i.v. (1×50 µg of anti Id) 24 hours after completion of the ten injections of bi- or oligospecific receptors. This single injection speeded up the elimination of the unbound bi- or oligospecific receptor molecules from the blood and increased the metabolism rate in liver and spleen.

It is possible on the basis of this manipulation to inject the chelate (EDTA-Y90) only 4 days after completion of the phase of penetration and binding of the bi- or oligospecific receptor. The following treatment regimen (for nude mice) is derived from these investigations

a) day 1–10, i.v. injection of 1×250 µg of bi- or oligospecific receptor each time

b) day 11, i.v. injection of 1×50 µg of anti Id

c) day 14, i.v. injection of a therapeutic dose of EDTA-Y90.

On the basis of comparative immunoscintigraphic data in nude mice and tumor patients, this regimen ought to be suitable for tumor therapy in humans too. However, the amounts to be injected in the human system are in a different range of magnitude. 10×5–10 g of bispecific receptor, 1×1 g of anti Id. Injection of the anti Id is not indispensable for therapy.

Annex 1a

Quantitative inhibition ELISA for MAbs by DTPA or EDTA complexes

Material: divisible 96-well polystyrene microtiter plates (U shape) type B, from Nunc, No. 4-60445

1) 50 µl of Y-benzyl-DTPA-HSA 19 conjugate with a concentration of 1 µg of conjugate per ml of PBS, pH 7.2, are pipetted into each well and incubated at room temperature (RT) overnight.

2) The supernatant is removed by aspiration and washed 3×with 0.05M tris citrate buffer, pH 7.4, (wash solution 1); (1×wash=introduce 250 µl of wash solution per well, leave to stand for 2 min and remove by aspiration).

3) If the microtiter plate is not required immediately, it is left to stand (with the opening underneath) on cellulose at RT overnight. The plate is then sealed in films with drying cartridges (from Gaplast, Postfach 529, 8100 Garmisch-Partenkirchen). The plates can be kept at +4° C. for at least 8 weeks under these conditions.

4) 250 µl of blocking solution are applied to each well and incubated at 37° C. for 30 min.

5) Preincubation of the diluted hybridoma supernatant with the competitor is carried out during the blocking (see Annex 1b).

6) 50 µl of the appropriately prediluted and preincubated hybridoma supernatants to be tested are applied to each well and incubated at RT for 30 min.

7) Washing 3×with wash solution 2 is subsequently carried out.

8) Subsequently 50 µl of goat anti-mouse IgG_1 antibodies which are labeled with alkaline phosphatase and have been diluted 1:500 in blocking solution are applied to each well and incubated at RT for 30 min.

9) Then washing 3×with wash solution for Enzygnost is carried out.

10) Subsequently 50 µl of 0.1 mMNADP are added.

11) Incubation at RT is then carried out for 30 min.

12) During the incubation with NADP, the amplification system is made up as follows: per plate 2 parts of INT and 1 part of PBS, pH 7.2, are mixed and 1 part of diaphorase and 1 part of ADH are pipetted in.

13) 50 µl of this system are placed in each well.

14) When there is a distinct change in color from transparent to red the reaction is stopped with 100 µl of a 0.1N H_2SO_4 solution per well.

15) The extinctions are measured at 492 nm in a TITERTEK® MULTISCAN. 50 µl of NADP with 50 µl of solution and 1200 µl of 0.1N H_2SO_4 are employed as blank.

NADP—Sigma order No. N-0505
INT—Sigma order No. I-8377
ADH—Sigma order No. A-3263
DIAPHORASE—Sigma order No. D-2381
Wash solution 2—Behring, order No. OSEW96 contains Tween/PBS

Blocking solution:

PBS, pH 7.2, is made 3% strength in casein by adding casein and stirring for 30 minutes, and is adjusted to pH 7.4. Particles are then removed by centrifugation at 4,000 rpm for 10'.

Diluted goat anti-mouse IgG_1 antibodies labeled with alkaline phosphatase (from Southern Biotechnology Associates, Cat. No. 1080-04).

Preparation of 0.1 mMNADP:

Dissolve 7.65 mg of NADP in 100 ml of 20 mM tris, 0.1 mM $MgSO_4$, pH 9.5; this solution can be stored at –20° C. for several months.

Preparation of INT (P-IODONITROTETRAZOLIUM Violet):

Dissolve 2.5 mg/ml of 30% ethanol in an ultrasonic bath; always make up fresh.

Preparation of diaphorase:

1 mg of diaphorase/ml of PBS, pH 7.2, is stored in portions at –20° C.

Preparation of alcohol dehydrogenase:

0.5 mg of ADH/ml of PBS, pH 7.2, are stored in portions at –20° C.

Annex 1b

Preincubation of the hybridoma supernatant with the competitor

The mouse IgG concentration in hybridoma supernatants can be determined using commercially available quantitative ELISA systems and is state of the art.

On the basis of the ELISA concentration determination, the hybridoma supernatants are diluted to 1.25 µg/ml in PBS without Ca^{++} and Mg^{++}.

Conversion from gram into mol:

50,000 g–1 mol of MAb

1.25×10^{-6} g–xmol

1.25 µg=x=8.33×10^{-2} mol

In order to have a 1+1 ratio of MAb and inhibitor, 10 µl of inhibitor with a concentration of 8.33×10^{-12} mol/200 µl, which is increased by a factor of 5, were added to 50 µl of hybridoma supernatant with a concentration of 8.33×10^{-12} mol/ml.

The hybridoma supernatant is incubated with 100,000-fold, 50,000-fold, 10,000-fold, 5,000-fold, 1,000-fold and 100-fold excess of competitor at RT for 30'. 50 µl of this are pipetted into the ELISA (see Annex 1a, no. 6).

Annex 1c

Production of the DTPA and EDTA complexes

The complexing constant of DTPA or EDTA to the metal ions depicted in Table I is extremely high so that complete saturation has to be expected on equimolar mixing of DTPA or EDTA with these metal ions. For this reason, the corresponding metal ions were incubated in a 3-fold molar excess with the DTPA or EDTA. As an example, 170 µl of a 10 mM cadmium sulfate solution in double-distilled water (see Annex 1d) were incubated with 30 µl of a 0.028 molar DTPA stock solution in double-distilled water at RT for 5'. Mixing 10 µl of this competitor solution with the hybridoma supernatant leads to a 100,000-fold excess of competitor over the MAb contained in the hybridoma supernatant. Lower competitor to MAb ratios were achieved by diluting the competitor solution in the particular salt ion solution appropriately for the desired molar excess (see Annex 1b).

Annex 1d

Source and relevant physicochemical parameters of the metal ions employed

MAb No.	DTPA-Y	DTPA	DTPA-Mn	DTPA-Cd	DTPA-Zn	DTPA-Cu
2050/174	10^4	10^3	10^2	10^2	5×10^3	5×10^3
2050/531	5×10^4	10^3	10^2	10^2	5×10^3	5×10^3
2050/532	5×10^4	10^3	10^2	10^2	5×10^3	5×10^3
2050/534	5×10^4	10^3	10^2	10^2	5×10^3	5×10^3
2050/535	10^4	10^3	10^2	10^2	10^3	10^3

MAb No.	DTPA-Pb	1,2-Diamino-ethane	Trans-aconitic acid			
2050/174	10^3	no inhibition up to 10^5	no inhibition up to 10^5	10^2	10^3	10^3
2050/531	5×10^3	no inhibition up to 10^5	no inhibition up to 10^5	10^3	10^3	10^3
2050/532	5×10^3	no inhibition up to 10^5	no inhibition up to 10^5	10^2	10^3	10^3
2050/534	5×10^3	no inhibition up to 10^5	no inhibition up to 10^5	10^2	10^3	10^2
2050/535	10^3	no inhibition up to 10^5	no inhibition up to 10^5	10^2	10^2	10^2

MAb No.	EDTA-Cd	EDTA-Zn	EDTA-Cu	EDTA-Pb
2050/174	10^3	10^3	10^3	5×10^3
2050/531	10^3	10^3	10^2	10^5
2050/532	10^5	10^3	5×10^3	10^5
2050/534	10^2	10^3	10^3	5×10^3
2050/535	10^2	10^2	10^2	10^2

Molar excess of competitor which leads to 50% inhibition of the binding to the solid-phase antigen.

TABLE 1

Peptide linkers with relevant nucleotide sequences

E P K S C G G E A A P A
+5' CTCTCTGCAGAGCCCAAATCTTGTGGCGGCGAGGCAGCTCCCGCAG
–3' GAGAGACGTCTCGGGTTTAGAACACCGCCGCTCCGTCGAGGGCGTC

A A P A A A A A G G Q V Q L Q E S
CTGCACCCGCAGCAGCCGCAGCAGGCGGGCAGGTCCAACTGCAGGAGAGC 3'
GACGTGGGCGTCGTCGGCGTCGTCCGCCCGTCCAGGTTGACGTCCTCTCG 5'

+ = coding strand
– = complementary strand

10 millimolar solutions of the following metal ions were prepared in double-distilled water:

Manganese chloride from Merck	MW 161.88 No. 5934	Mn ion radious: 80 pm
Cadmium sulfate Riedel de Haer	MW 256.5 No. 31145	Cd ion radious: 97 pm
Zinc chloride from Merck	MW 136.28 No. 8816	Zn ion radius: 74 pm
Copper sulfate from Riedel de Haen	MW 159.61 No. 31294	Cu ion radius: 96 pm
Yttrium chloride from Aldrich	MW 303.36 No. 20,491–9	Y ion radius: 92 pm
Lead(II) nitrate from Riedel de Haen	MW 331.20 No. 31137	Pb ion radius: 120 pm

Annex 1e

Quantitative assay of inhibition of MAb by DTPA and EDTA

TABLE 6

Mutagenic oligonucleotides:

1) 5'CTTACCTGGG.CATGCCCCGA.GCTCCCGTGG.GCATGT3'
2) 5'AGTGGGGTTT.TCAGCTCTGCAGAG3'

TABLE 7

Mutated hinge exon:

E\/L\/K\/T\/P\/L\/G\/D\/T\/T\/H\/T\/C
AGAGCTCAAA.ACCCCACTTG.GTGACACAAC.TCACACATGC
↓
G

TABLE 7-continued

Mutated hinge exon:

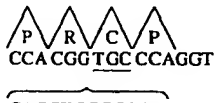


TABLE 8

Jun I oligonucleotide

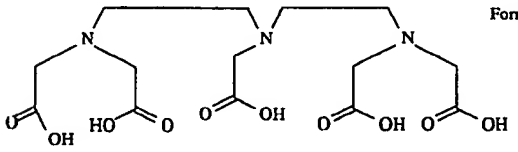
5'CTACGCTCGG.CTAGAGGAAA.AAGTGAAAAC.
CTTGAAAGCG.CAAAACTCCG.AGCTGGCATC.
CACGGCCAAC.ATGCTCAGGG.AACAGGTGGC.
ACAGCTTAAG.CAGAAAGTCA.TGAACCACCG.
ACCTGCATB3'.

Jun II oligonucleotide

5'CAGGTCGGTG.GTTCATGACT.TTCTGCTTAA.
GCTGTGCCAC.CTGTTCCCTG.AGCATGTTGG.
CCGTGGATGC.CAGCTCGGAG.TTTTGCGCTT.
TCAAGGTTTT.CACTTTTTCC.TCTAGCCGAG.
CGATGAGCT3'.

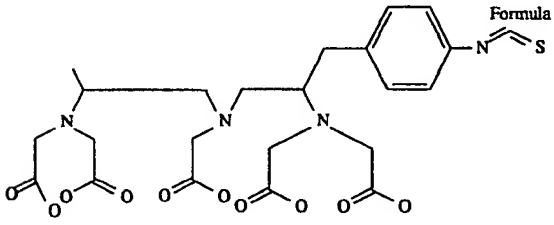
DTPA

Formula 1



Isothiocyanatobenzyl-DTPA

Formula 2



We claim:

1. A bispecific or oligospecific mono- or oligovalent receptor produced by gene manipulation comprising:

a) a VH and a CH1 region of a first antibody having an antigen binding specificity;

b) a VH and a CH1 region of a second antibody having an antigen binding specificity, wherein the antigen binding specificity of said first antibody is different from the antigen binding specificity of said second antibody; and

c) a polypeptide spacer that links the CH1 region of said first antibody to the VH region of said second antibody without impeding association with light chains of said antibodies or antigen binding.

2. A receptor as claimed in claim 1, wherein said receptor further comprises light chains of said first and said second antibodies.

3. A diagnostic composition comprising a receptor as claimed in claim 2.

4. A receptor as claimed in claim 2, wherein said first antibody binds to animal or human tumor-associated antigens.

5. A receptor as claimed in claim 2, wherein said receptor has catalytic or enzymatic activity.

6. A receptor as claimed in claim 2, wherein said first antibody binds to animal or human tumor-associated antigens and said second antibody binds to a chelate.

7. A receptor as claimed in claim 2, wherein a variable region of said first antibody comprises an amino acid sequence of V_H or V_K selected from the group consisting of an amino acid sequence of V_H or V_K of FIG. 22, FIG. 23, FIG. 24, and FIG. 25.

8. A receptor as claimed in claim 4, wherein a variable region of said first antibody comprises an amino acid sequence of V_H or V_K selected from the group consisting of an amino acid sequence of V_H or V_K of FIG. 22, FIG. 23, FIG. 24, and FIG. 25.

9. A receptor as claimed in claim 5, wherein a variable region of said first antibody comprises an amino acid sequence of V_H or V_K selected from the group consisting of an amino acid sequence of V_H or V_K of FIG. 22, FIG. 23, FIG. 24, and FIG. 25.

10. A receptor as claimed in claim 6, wherein a variable region of said first antibody comprises an amino acid sequence of V_H or V_K selected from the group consisting of an amino acid sequence of V_H or V_K of FIG. 22, FIG. 23, FIG. 24, and FIG. 25.

* * * * *

F

Section Review

Biologicals & Immunologicals

The use of immunoconjugates in cancer therapy

Maria-Ana Ghetie, Victor Ghetie & Ellen S Vitetta

Department of Microbiology and Cancer Immunobiology Center,
University of Texas Southwestern Medical Center, 6000 Harry Hines, Dallas, Texas 75235, USA

Those immunoconjugates that have entered clinical trials (i.e., immunotoxins, radiolabelled and bispecific antibodies) have shown promising antitumour activity in haematopoietic tumours (lymphomas/leukaemias). To eliminate large solid tumours, immunoconjugates will require changes in both the antibody and effector moieties since these tumours are poorly vascularised and rarely express tumour-specific antigens. This review describes improvements which may have a major impact on the next generation of immunoconjugates.

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Introduction

Immunoconjugates (IC) are cell-targeting molecules, such as monoclonal antibodies, cytokines or soluble receptors that have been genetically or biochemically coupled to cytotoxic moieties, such as toxins (or their subunits), radioisotopes, drugs, enzymes which can activate prodrugs, or effector cell recruiting structures. From the multitude of ICs with *in vitro* and *in vivo* antitumour activity, three types have entered clinical trials in humans: immunotoxins (ITs), radiolabelled antibodies, and bispecific antibodies (BsAbs).

ICs for the Therapy of Haematologic Malignancies

The ICs used to date in Phase I clinical trials have been directed against either haematopoietic tumours, such as lymphomas/leukaemias, or solid tumours, such as melanomas and colorectal or ovarian carcinomas (Table 1) [1-38]. The results of early clinical trials have suggested that solid tumours are less suitable targets for the present generation of ICs than lymphomas or leukaemias. This conclusion is also supported by the results of numerous studies in IC-treated nude or SCID mice xenografted with human tumours (Table 1). Results of clinical trials have also indicated that the immunogenicity of ICs is not a major obstacle in immunosuppressed lymphoma patients and that a significant number of patients can be treated with several courses of therapy before they generate antibodies [39]. To date, the antitumour activity of [^{131}I]-MAbs is greater than that of MAb-toxins (ITs) in lymphoma patients with large tumour burdens [40-42]. The difference between the therapeutic effect of ITs and radiolabelled [^{131}I]-MAbs is undoubtedly related to their mechanisms of action and the types of tumours which are most amenable to treatment. Hence, [^{131}I]-MAbs have excellent antitumour activity in large tumours since high levels of radioactivity are concentrated in the tumour mass; bystander tumour cells which may lack the targeting antigen are also killed [43]. In addition, patients likely to respond to [^{131}I]-MAbs can be identified based on preliminary radioimaging studies using small amounts of the [^{131}I]-MAbs [44].

Abbreviations

BsAb: Bispecific antibody; DT: Diphtheria toxin; FN: Fibronectin; HUVEC: Human umbilical vein cells; IC: Immunoconjugate; IT: Immunotoxin; MAb: Monoclonal antibody; MTD: Maximum tolerated dose; PAP: Pokeweed antiviral protein; PE: *Pseudomonas* exotoxin; PEG: Polyethylene glycol; SEA: Staphylococcal enterotoxin A; VLS: Vascular leak syndrome.

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Unlike [^{131}I]-MAbs, ITs work best to kill small numbers of tumour cells in the setting of minimal disease. ITs are highly potent but they must bind to every cell which they kill; there is no significant bystander effect. Results of Phase I and II clinical trials with ITs suggest that they should be most successful in an adjuvant setting for treating minimal disease [45]. Indeed, radiolabelled MAbs administered to patients with bulky disease followed by ITs to treat minimal residual disease might be the best way to use these modalities. The efficacy of ITs might be further improved by combining ITs containing antibodies with different specificities. Indeed, 'cocktails' of ITs have excellent antitumour activity in SCID mice with human lymphomas [2,46,47] and are curative in combination with chemotherapy [8,12,15,46,48]. It remains to be determined whether similar results will be achieved in patients with primary tumours.

ICs for the Therapy of Solid Tumours

Although ITs may work well on small numbers of metastatic cells from solid tumours, ICs with the ability to eliminate larger solid tumours will require changes in both the antibody and the toxic moieties since large solid tumours are not well vascularised and rarely express tumour specific antigens. ICs presently under development are currently being tested in animal models and early results suggest that they might be more effective in treating solid tumours.

The Antibody Moiety

It would be desirable to treat large solid tumours with multiple courses of therapy. The main obstacle for the repeated administration of ICs is related to both the rodent origin of the antibody and its size [49]. The development of an antibody response against the murine MAb may lead to the rapid neutralisation of the IC and, infrequently, harmful allergic reactions. Humanised mouse antibodies have lower immunogenicity and a higher residence time; these features might be useful for constructing improved radiolabelled MAbs to treat both lymphoma and solid tumours [50]. The use of humanised antibodies to construct ITs is less attractive since the current generation of plant and bacterial toxins will remain immunogenic and will have shorter half-lives *in vivo*.

Size

Decreasing the size of the antibody might facilitate tumour penetration. In this regard, antibody moieties (150 kDa) have been reduced to 25 kDa by using Fv fragments obtained by genetic engineering [51]. However, by removing the Fc portion one also removes the site necessary for persistence of the IT in the circulation [52]. Consequently, antibody fragments devoid of their Fc portion have a short half-life and are rapidly eliminated from the circulation [50]. Thus, the benefit of smaller MAbs would be lost unless continuous infusion regimens were used. To prolong the persistence of bolus doses of ITs in the circulation, additional structural changes could be made to protect the IT from rapid clearance. Attempts to attach polyethylene glycol (PEG) residues to antibody fragments (or to the toxin moiety) have resulted in longer half-lives and enhanced accumulation of antibodies in the tumours of nude mice [53]. Another approach has been to splice a CH_2 domain into ITs constructed with an Fab fragment of antibody. These ITs have a longer residence time even though their half-life is shorter than that of an IT prepared with an intact antibody [54]. Attaching the amino acid residues from the CH_2CH_3 domain interface might therefore be a better solution for constructing smaller ITs with longer half-lives [55]. A longer half-life is necessary since the penetration of solid tumours requires several days and half-lives shorter than this might represent an obstacle for effective therapy [56].

Affinity

It has been suggested that the uptake and penetration of antibody into solid tumours might be improved by using MAbs of higher affinity/avidity [57]. Arguing against this, however, is the finding that tumour penetration may in fact be reduced when using antibodies of higher affinity [58]. Nevertheless, the most important parameters for any antibody-based IC are its concentration and rate of association with its cell-associated antigen [59]. A slow association rate may be a drawback in clinical trials if the half-life of association with the tumour cells is shorter than the half-life of removal of the IT from the circulation.

Internalisation

Antibodies which react with high density cell surface antigens that are rapidly internalised to the optimal intracellular compartment would make effective ITs and drug conjugates. Less effective internalisation would be more desirable for radiolabelled ICs or for those ICs requiring the conversion of prodrugs or the binding of effector cells. It should be noted that, in the case of ITs, potency is highly dependent upon intracellular routing rather than the number of internalised molecules *per se* [60]. Thus, the kinetics of cellular routing of several ITs correlated with the kinetics of protein synthesis inhibition indicating that the concentration of the intracellular IT depends not only on the amount of IT internalised, but also on its rate of

Table 1: Therapeutic effect of ITs in both tumour-bearing SCID/nude mice and patients.

Immunotoxin[a]			Disease	Therapeutic effect[b]				Antibody response[c]	Ref.
				PR(%)		CR(%)			
Antibody	Specificity	Toxin		Mice	Humans	Mice	Humans		
RFB4	anti-CD22	dgRTA	B-lymphoma	100	25	0	2	59/19	[1-5]
HD37	anti-CD19	dgRTA	B-lymphoma	100	3	0	3	33/10	[2,3,6]
B4	anti-CD19	bR	B-lymphoma	100	29	0	13	68/34	[7,11]
B-43	anti-CD19	PAP	B-leukaemia	7	6	65	24	17/NR	[12-15]
RFT5	anti-CD25	dgRTA	Hodgkin's lymphoma	0	8	95	8	16/NR	[16-19]
Ber-H2	anti-CD30	SAP	Hodgkin's lymphoma	0	43	80	0	16/NR	[20,21]
B3	anti-Le^y	PE 38/40	Ovarian carcinoma	0	6	100	0	32/28	[22-25]
OVB3	anti-OVB3	PE	Ovarian carcinoma	100	0	0	0	23/23	[26-29]
XomaZyme-791	anti-gp 72	RTA	Colorectal and ovarian carcinoma	100	12	0	0	17/16	[30,32]
XomaZyme-MEL	anti-MAA	RTA	Melanoma	100	11	0	1	65/60	[33,38]

[a] bR: Blocked ricin toxin; dgRTA: Deglycosylated ricin A chain; gp72: Glycoprotein present on osteogenic sarcoma, ovarian and colorectal tumours; Le^y: Le^y-related carbohydrate; MAA: Melanoma-associated antigen; OVB3: Not characterised antigen present on adenocarcinoma of ovary, breast and colon; PAP: Pokeweek antiviral protein; PE: *Pseudomonas* exotoxin; RTA: Ricin A chain; SAP: Saporin.

[b] PR: Partial response. In mice this was considered to be any extension of survival time which was significantly different in comparison with untreated mice; CR: Complete response. In mice this is tumour-free survival for up to one year. All tumours in mice were early tumours except $CD30^+$ Hodgkin lymphoma where it induced a PR in 30% of the animals with late tumour.

[c] Total number of patients treated over number of patients with an immune response to either the antibody or the toxin moiety of IT. NR: Not reported.

intracellular degradation. This rate, in turn, depends upon both the rate of translocation of the toxin from the Golgi to the cytosol and on the rate of inactivation of the substrate by the toxin [61]. The importance of epitope recognition by the antibody on the potency of an IT has also been pointed out by the fact that ITs containing antibody which recognise epitopes proximal to the plasma membrane often make more effective ITs than those directed against epitopes more distal to the plasma membrane; the latter are probably routed to lysosome [62].

Targeting

In the case of solid tumours, antibodies could also be targeted to antigens present on the endothelial cells of the vasculature within the tumour. Vascular endothelial cells are directly accessible to the blood and are therefore easily reached by ITs. The ITs could destroy (or infarct) the vasculature of solid tumours, thereby depriving tumour cells of a blood supply. This approach also has a built-in amplification mechanism since thousands of tumour cells rely on each capillary for nutrients and oxygen. Hence, even incomplete damage to the vasculature should result in massive tumour destruction. These ITs need not penetrate the tumour mass or be in physical contact with the tumour cells to exert their tumouricidal effect. A model system in mice was developed using a IFNγ-transfected neuroblastoma cell line which induced expression of an activation antigen in the endothelial cells of the tumour vessels but not the normal endothelial cells elsewhere [63,64]. Treatment of the tumours with an IT directed against this antigen resulted in marked tumour regressions following haemorrhagic necrosis [64]. The search for antibodies which react with human endothelial cells in neoplastic but not normal tissues is underway [65]. The effector molecule of such ITs can be the ricin A chain or human coagulation-initiating proteins which trigger the coagulation cascade and induce thrombosis of the tumour vasculature resulting in massive tumour cell killing [66]. Human tissue factors also have the advantage of not being immunogenic.

The Toxin Moiety

The toxins which have been used to construct ITs for clinical trials (e.g., *Pseudomonas* exotoxin (PE), ricin, pokeweed antiviral protein (PAP), saporin and diphtheria toxin (DT)) are probably no more or less effective than those awaiting clinical testing (gelonin, abrin, mormodin, mitogilline, etc.) but this remains to be determined (Table 2). IT-bound toxins used in clinical trials have three features in common:

- the ability to kill targeted cells after internalisation;
- immunogenicity; and
- some normal cell damage, usually resulting in vascular leak syndrome (VLS) and/or liver damage.

Plant toxins

To improve cytotoxic activity and to decrease non-specific toxicity and immunogenicity, toxins have been modified to delete those portions responsible for non-specific toxicity and immunogenicity. In the case of plant toxins, size has been decreased by eliminating the subunit responsible for non-specific binding (e.g., the B chain of ricin or abrin) or by using toxins which are normally synthesised as single chains (e.g., PAP, saporin). Unfortunately, since the translocation site of ricin appears to reside in the cell-binding B chain [67] when ricin A-chain is used to construct ITs the resulting constructs do not always have the desired cytotoxicity [68]. As a result, some investigators have used 'blocked' ricin to construct ITs with better cytotoxic activity despite their larger size (60 kDa *vs.* 30 kDa) and unwanted non-specific toxicity and immunogenicity (due to B chain) [69]. The size of single chain plant toxins cannot be further decreased by removing the nonessential portions since the toxic site is conformational and involves amino acid residues scattered over a long stretch of the polypeptide chain [70]. Unfortunately, these modified toxins still contain portions responsible for unwanted side-effects, like VLS.

To facilitate the translocation of ITs, the DNA encoding a viral peptide of 25 amino acids residues has been spliced to the DNA encoding ricin A chain [71]. The resulting expressed construct was 10-fold less toxic as a free A chain to leukaemic cells than its unmodified counterpart, but showed 10 - 20 times greater cell-killing when targeted to transferrin receptors. These results clearly demonstrated that the specific cytotoxic efficacy (therapeutic window) of ITs containing ricin A chain could be increased by > 100-fold [71]. Whether such constructs will be safe, exhibit reduced toxicity and/or have greater efficacy in humans, remains to be determined.

A different approach for increasing the cytotoxicity of ITs is to increase the toxin/antibody ratio by linking the antibody to more than one molecule of toxin [72]. In an experiment utilising anti-CD22 antibody conjugated to one or two molecules of deglycosylated ricin A chain, non-specific toxicity (LD_{50}) remained the same but the specific cytotoxicity *in vitro* and the antitumour activity *in vivo* were greatly improved [72,73].

These results have been confirmed using several ITs with different specificities, indicating that increasing the toxin/antibody ratio might, in some cases, improve the cytotoxic activity of certain ITs without increasing their non-specific toxicity [74,75].

Bacterial toxins

The molecular mass of bacterial toxins (i.e., PE) has been decreased by deleting the cell binding site domain, but the remainder of the molecule cannot be modified since it contains both the toxic domain and > 100 amino acid residues of the translocation domain (which allows the toxin to reach the cytosol of targeted cells) [24]. The smallest active fragment of PE has a molecular mass of 35 kDa *vs.* 66 kDa (the molecular mass of the intact PE) and can be considered an analogue of ricin A-chain (molecular mass 30 kDa) containing a free SH group in its C-terminal portion [76].

To increase the cytoplasmic delivery of ITs containing PE, an endoplasmic reticulum-retention sequence (KDEL) was genetically engineered into the molecule [24]. The cytotoxic activity of ITs containing KDEL-truncated-PE (PE38 or PE40) is increased both *in vitro* and in SCID mice xenografted with various human tumours [77]. The addition of a KDEL peptide sequence to the A-chain of ricin toxin results in increased cytotoxicity when the mutant A-chain is reassociated with its native B-chain [78], but not when ITs are prepared with this mutant A chain ([79]; Lark and Vitetta, unpublished results).

Another possibility for enhancing retention and internalisation of ITs is to introduce amphipathic peptides (such as the tetrapeptide GALA) into either the toxin or antibody, since GALA interacts with membrane bilayers [80]. Fab fragments containing GALA have indeed shown better tumour localisation than the same fragments of antibody without GALA [80].

Table 2: The most frequently used toxins for the preparation of ITs.

Name of toxin and abbreviation	Source	Genes/Species	Molecular mass (kDa)
Ricin (R), blocked ricin (bR)	Plant	*Ricinus communis* (castor bean) seeds	65, 72.5
Ricin A chain (RTA)	Plant		30
Deglycosylated RTA (dgRTA)	Plant		30
Abrin	Plant	*Abrus precatorius* (Jequirity bean) seeds	65
Abrin A chain	Plant		30
Pokeweed antiviral protein (PAP)	Plant	*Phytolacca americana* (pokeweed) leaves	30
Saporin (SAP)	Plant	*Saponaria officinalis* (soapwort) seeds	29.5
Gelonin	Plant	*Gelonium multiflorum* seeds	30
Momordin	Plant	*Momordica charantra* (buttergourd) seeds	31
Viscumin	Plant	*Viscum album* (mistletoe) leaves	65
Viscumin A chain	Plant		32
Barley toxin	Plant	*Hordeum vulgare* (barley) seeds	30
Trichosanthin	Plant	*Trichosantes kirilowli* roots and seeds	25.6
Diphtheria toxin (DT)	Bacteria	*Corynebacterium diphterii*	58.4
Recombinant Diphtheria toxin (CRM45)	Bacteria		45
Pseudomonas exotoxin (PE)	Bacteria	*Pseudomonas aeruginosa*	66
Recombinant *Pseudomonas* exotoxin (PE40)	Bacteria		40
α-Sarcin, mitogillin	Fungi	*Aspergillus giganteus*	16.5, 16.8
Restrictocin	Fungi	*Aspergillus restrictus*	16.8

Side-effects of toxins

In most clinical trials with ITs, VLS is the dose-limiting toxicity [45]. This syndrome is manifested by decreased serum albumin, accumulation of fluid in the interstitial spaces, weight gain, oedema and accumulation of fluid in lungs and pericardium [3,4]. VLS has made it difficult to reach blood concentrations of ITs necessary to achieve maximum therapeutic responses. VLS might be due to the binding of plant toxins to vascular endothelial cells, subsequent killing of these cells and damage to the capillary vessels [81].

An *in vitro* model of VLS has been developed to document the morphological and biochemical modifications of monolayers of human umbilical vein cells (HUVEC) caused by plant toxins and ITs containing plant toxins [81]. The vascular toxicity of ITs containing ricin A chain may reside in the ability of the toxin moiety to interact (directly or in conjunction with fibronectin (FN)) with receptors on the endothelial cells [82]. Recently, it has been shown that ITs containing PE may also induce VLS in patients but this effect was the result of the binding of the antibody (B3) to the endothelial cells and not of the toxin [83]. However, a single chain IT containing PE40 has induced VLS-like symptoms in rats (but not in mice), suggesting that PE, like RTA, might damage vascular endothelial cells directly [84].

Several strategies might be considered to diminish IT-mediated VLS. The most practical treatment might involve anti-inflammatory drugs to reduce oedema (e.g., corticosteroids). Unfortunately, this is not always successful in a clinical setting. In the case of ricin A chain-containing ITs, the concomitant administration of FN might prevent VLS, although this has not yet been attempted. Finally, it might be possible to eliminate the region of the toxin responsible for its binding to endothelial cells without impairing its ability to inhibit protein synthesis.

Toxic moieties not requiring internalisation

Toxic moieties which kill target cells in the absence of internalisation represent an attractive option for constructing ICs since the degree to which ICs are internalised and effectively routed is unpredictable. Presently, there are four categories of such toxic moieties including cytolytic, prodrug/drug converting, killer cell recruiting effectors and radionuclides.

Cytolytic toxins

Phospholipase C [85], pore forming proteins (e.g., staphylococcal α-haemolysin) [86] and some of the complement components [87] have already been used to construct ITs which can lyse target cells *in vitro*. These immunolysins have enhanced non-specific toxicity due to their ability to bind to and kill life-sustaining cells (e.g., erythrocytes) [87]. Therefore, enzymes with cytolytic activity will have to be altered to remove the cell binding site responsible for non-specific toxicity. This will only be feasible if the active site of the enzyme and the cell binding domain reside in different portions of the molecule. In this regard, preliminary data indicate that the removal of the cell-binding site might be possible for at least some pore-forming toxins [88].

Prodrug/drug converting enzymes

Enzymes which generate low molecular mass cytotoxic drugs from relatively non-cytotoxic precursors (prodrugs) have also been used to construct ICs [89]. Once bound to tumour cells, these antibody-enzyme conjugates convert the prodrugs into cytotoxic compounds of low molecular mass [89]. These compounds then diffuse into the tumour mass and kill tumour cells which are not in physical contact with the conjugate.

The advantage of this approach is that the targeted enzyme can greatly amplify the number of drug molecules delivered to each tumour cell. Furthermore, the cytotoxic effect is not restricted to tumour cells which bind the conjugate since the drug is released extracellularly where it diffuses to neighbouring tumour cells. Several studies have shown that the antibody-enzyme conjugate/prodrug combination is more effective than systemic drug therapy [90-92]. For example, a humanised disulphide-stabilised Fv fragment fused to beta-lactamase activated the cephalosporin-doxorubicin prodrug and had good antitumour activity in mice [92]. The drawback of this approach is that the enzymes are of bacterial or animal origin and, like toxins, are immunogenic in humans. It might, however, be possible to use enzymes of human origin coupled to humanised antibodies [87] or to develop humanised catalytic antibodies which bind to tumour-associated antigens.

Effector cell recruiting structures

Other effector molecules which completely change the *modus operandi* of the immunoconjugate are superantigens or cell-binding BsAbs. Both superantigens and BsAbs can bridge tumour cells to effector cells and thereby lead to cytolysis.

Superantigens

The superantigen staphylococcal enterotoxin A (SEA), has been spliced to an Fab' fragment of an antibody [93-95]. SEA binds to a variety of different MHC Class II$^+$ T-cells which are subsequently activated to proliferate, produce cytokines and kill target cells [96]. Antibody-SEA ICs bridge target cells expressing the molecule which the MAb recognises to cytotoxic T-lymphocytes (CTLs). Fab'-SEA therapy in humanised SCID mice xenografted with human colon carcinomas or transfected melanoma cells induced massive T-cell infiltration in the tumour and significant suppression of tumour growth [93-95]. In one instance, the antibody-SEA conjugate was more effective than BsAbs reacting with the TCR-CD3 complex and the tumour-associated antigen [97]. Unfortunately, the antibody-superantigen conjugate induces the release of cytokines which cause systemic toxicity [95]. To eliminate this problem; it will be necessary to alter SEA to diminish its ability to induce cytokine release [98]. Finally, the immunogenicity of superantigens might represent a significant obstacle to the repeated administration of ICs to patients.

BsAbs

BsAbs can be generated by:

- coupling two antibody molecules with different specificities using cross linking agents [99]; or
- fusing two hybridoma cell lines to produce a hybrid-hybridoma (quadroma) that secretes BsAb [99].

Like SEA, BsAbs bind to both tumour cells and effector cells and those directed against tumour antigens and CD3$^+$ cells (T-cells) or (CD64) FcRI$^+$ cells have given encouraging antitumour effects in Phase I clinical studies [100,101].

To increase the stability and penetration of BsAbs, small BsAbs have been produced by genetic engineering [99]. Two Fv regions with different specificities were coupled to each other by a helical peptide linker [99]. The advantage of these single chain BsAbs over 'classical' BsAbs is their better penetration, stability and decreased immunogenicity [99]. Single chain BsAbs have been expressed in both bacteria and mammalian cells. As mentioned earlier, the disadvantage of using Fv fragments is their short $T_{1/2}$ *in vivo*. In this regard, Fv fragments of an anti-L6 tumour associated antigen have been linked to the Fv-CH_2-CH_3 of an anti-human CD3 [102]. The resulting BsAb promoted adhesion

between human T-cells and L6$^+$ tumour cells and stimulated the T-cells to proliferate and mediate killing of L6$^+$ tumour cells. The $T_{1/2}$ of this construct was not reported but it would be predicted to be considerably longer than that of Fv BsAb lacking CH_2-CH_3 domains.

Radionuclides

^{131}I is the most frequently used radionuclide for constructing radiolabelled ICs [103,104]. The main disadvantage of ^{131}I is that it has a high abundance (81%) and high energy (346 KeV) gamma-emission which induce non-specific irradiation of other organs. Moreover there is a 'wash out' of the radioiodine from the tumour due to the dehalogenation of the iodotyrosine residues [103]. The released low molecular weight radioiodine species concentrate in the thyroid [103].

As a potential replacements for ^{131}I, pure beta-emitters (Yttrium-90) or beta-emitters with low abundance and energy gamma-radiation (Rhenium-186, Indium-111, etc.) have already been tested in animal models and clinical trials [103]. These second generation radionuclides have better radiophysical characteristics than ^{131}I and better retention at the tumour level but their $T_{1/2}$s are much shorter (^{90}Y = 64 h, ^{186}Rh = 90 h) or similar (^{111}In = 7.5 days) to that of ^{131}I [103]. Furthermore, the chemistry of coupling metallic isotopes to proteins requires the use of intermediary chelating ligands (e.g., DTPA or DOTA) yielding conjugates which are less stable than those prepared with [^{131}I]-antibody [103]. The chemical instability of the former may lead to the release of the radioisotope into the circulation with subsequent localisation in bone. This drawback is currently being addressed by constructing antibody-radiometal conjugates with better stability and retention [103].

The optimal isotope for an IC should be a beta-emitter with a relatively long $T_{1/2}$ to take advantage of an extended residence time of the antibody in the tumour. For example, Phosphorus-32 (^{32}P) is a pure beta-emitter with high specific activity and with a $T_{1/2}$ of 14 days. Despite the potential advantages of ^{32}P, its development has been hindered by difficulties in the labelling chemistry. Recently, procedures to conjugate [^{32}P]-ATP to antibody have been described [105]. Thus, ^{32}P has been conjugated to a heptapeptide (Trp-Arg-Arg-Ala-Ser-Leu-Gly) fused to the hinge region of Fab' anti-CEA [106]. The conjugate did, however, release free ^{32}P in human serum at 37°C. The use of another linkage (thiophosphate ester) may result in even more stable [^{32}P]-antibody ICs. Co-administration of cold phosphate might minimise bone uptake of ^{32}P. The [^{32}P]-labeled humanised Fab or IgG antibody would also be expected to have a longer residence time and an improved therapeutic index.

Toxic moieties requiring internalisation

Anti-neoplastic drugs

Historically, the cytotoxicity of Ab-drug ICs has been no greater than that of the unconjugated drugs, due to two factors:

- the drug acts stoichiometrically and not catalytically; and
- the type of the chemical linkage between the drug and antibody does not allow efficient release of the drug into the cytosol [107].

These limitations have been partially overcome by either replacing the current drugs with synthetic compounds endowed with a 1000-fold greater cytotoxicity [108,109] and/or by using better linkers which release the active drug inside the cell [110]. The new drugs (e.g., calicheamicins and maytansinoids) have greater systemic toxicity which precludes their use at therapeutic doses, but when these drugs are bound to antibodies at a low molar ratio they lose their non-specific toxicity resulting in a significant therapeutic window for treating solid tumours in mice [108,109]. In mice, the LD_{50}s and IC_{50}s of some of these Ab-drug ICs have been comparable to those of ITs containing ricin A chain. Their antitumour activity in nude mice with human breast tumours has been excellent [108,109].

The importance of the linker has been established by using an acid-labile hydrazone bond, which liberates the internalised drug into an acidic environment, to prepare a doxorubicin-MAb IC [110]. An IC containing a chimeric human/murine monoclonal antibody that targets an antigen expressed at the surface of many human carcinomas induced cures of both subcutaneous and metastatic tumours [110]. The main advantage of using this novel type of IT *vs.* a classical one might reside in the lower immunogenicity of these drugs (molecular weight < 2000).

Inhibitors of signal transmission

Another drug which is devoid of immunogenicity and which kills the target cells through a different mechanism (involving the inhibition of tyrosine kinases) is genistein (molecular weight 300). Genistein is an isoflavone which inhibits tumour invasion and metastasis [111]. This compound has been chemically coupled to an anti-CD19 antibody and the IC used selectively to inhibit CD19-associated tyrosine kinases and trigger rapid apoptotic cell death in human lymphoma cells [112,113]. Thus, treatment of either human leukaemia cells (NALM-6) or Burkitt's lymphoma cells (RAMOS) in SCID mice resulted in 120-day survival rates of 100% and 70%, respectively. In contrast to

anti-cancer drugs, the anti-CD19-genistein conjugates lack systemic toxicity (MTD > 250 mg per mouse) [112,113]. Therefore, the therapeutic window was much wider than that of any antibody-drug IC. The application of genistein (or other inhibitors of signalling)-containing ICs might be limited to tumour cells expressing antigens associated with tyrosine kinases.

Humanised toxins

Humanised toxins were utilised in ITs after the toxicity of human proteins belonging to the ribonuclease A family was demonstrated. Fusion proteins containing antibody anti-transferrin receptor and human RNases have shown some cytotoxic activity *in vitro*, but their future use as antitumour agents might be impaired by the presence of ribonuclease inhibitors, present in the cell cytosol, which block toxicity [87].

Conclusion

To date, ICs have performed most effectively in the treatment of lymphomas. While [^{131}I]-Abs work best in bulky tumours, ITs appear promising for treating in minimal disease. A combination of these agents (± conventional therapy) could be highly effective in newly diagnosed disease. In this regard, the excellent results obtained in animal models using combination therapy with ITs and chemotherapy have been encouraging [8,46,48,114-116].

Besides refining the present generation of ITs, many new ITs against solid tumours are being tested in preclinical trials in animals to explore the possibility of improving their penetration and cytotoxicity. Thus, antibodies have been miniaturised and humanised with the goal of increasing penetration and/or reducing the anti-mouse IgG antibody response. ICs which do not require tumour penetration because they act on cells sustaining tumour growth (e.g., endothelial cells) are also being developed. The cytotoxic moieties of 'classical' ICs (e.g., toxins) have been replaced with a series of compounds endowed with the ability to kill tumour cells by other mechanisms. Some of these agents are not immunogenic (anti-neoplastic drugs), others do not require cellular internalisation (cytolysins, prodrug/drug converting enzymes, superantigens) and some act by inhibiting signal transduction (genistein). Early preclinical results obtained with this new generation of IC offer promise for the future.

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References to Primary Literature

Papers of special interest have been highlighted as:
- • of interest
- •• of considerable interest

1. GHETIE M-A, RICHARDSON J, TUCKER T, JONES D, UHR JW, VITETTA ES: **Antitumor activity of Fab' and IgG-anti-CD22 immunotoxins in disseminated human B lymphoma grown in mice with severe combined immunodeficiency disease: effect on tumor cells in extranodal sites.** *Cancer Res.* (1991) 51:5876-5880.

2. GHETIE M-A, TUCKER K, RICHARDSON J, UHR JW, VITETTA ES: **The antitumor activity of an anti-CD22 immunotoxin in SCID mice with disseminated Daudi lymphoma is enhanced by either an anti-CD19 antibody or an anti-CD19 immunotoxin.** *Blood* (1992) 80(9):2315-2320.

3. VITETTA ES, STONE M, AMLOT P, FAY J, MAY R, TILL M, NEWMAN J, CLARK P, COLLINS R, CUNNINGHAM D, GHETIE V, UHR JW, THORPE PE: **Phase I immunotoxin trial in patients with B-cell lymphoma.** *Cancer Res.* (1991) 51:4052-4058.

4. AMLOT PL, STONE MJ, CUNNINGHAM D, FAY J, NEWMAN J, COLLINS R, MAY R, MCCARTHY M, RICHARDSON J, GHETIE V, RAMILO O, THORPE PE, UHR JW, VITETTA ES: **A Phase I study of an anti-CD22-deglycosylated ricin A chain immunotoxin in the treatment of B-cell lymphomas resistant to conventional therapy.** *Blood* (1993) 82(9):2624-2633.

5. SAUSVILLE EA, HEADLEE D, STETLER-STEVENSON M, JAFFE ES, SOLOMON D, FIGG WD, HERDT J, KOPP WC, RAGER H, STEINBERG SM, GHETIE V, SCHINDLER J, UHR JW, WITTES RE, VITETTA ES: **Continuous infusion of the anti-CD22 immunotoxin IgG-RFB4-SMPT-dgA in patients with B-cell lymphoma: a Phase I study.** *Blood* (1995) 85(12):3457-3465.

6. SAUSVILLE EA, HEADLEE D, STETLER-STEVENSON M, JAFFE E, FIGG W, AMLOT P, KAPLAN L, STONE M, WITTES R, GHETIE V, SCHINDLER J, UHR JW, VITETTA ES: **Deglycosylated ricin-A chain immunotoxins in the treatment of B-cell non-Hodgkin's lymphoma.** *Fourth International Symposium on Immunotoxins* (1995). Abstract #156.

7. SHAH SA, HALLORAN PM, FERRIS CA, LEVINE BA, BOURRET LA, GOLDMACHER VS, BLÄTTLER WA: **Anti-B4-blocked ricin immunotoxin shows therapeutic efficacy in four different SCID mouse tumor models.** *Cancer Res.* (1993) 53:1360-1367.

8. O'CONNOR R, LIU C, FERRIS CA, GUILD BC, TEICHER BA, CORVI C, LIU Y, ARCECI RJ, GOLDMACHER VS, LAMBERT JM, BLÄTTLER WA: **Anti-B4-blocked ricin synergizes with doxorubicin and etoposide on multidrug-resistant and drug-sensitive tumors.** *Blood* (1995) 86(11):4286-4294.

9. GROSSBARD ML, FREEDMAN AS, RITZ J, CORAL F, GOLDMACHER VS, ELISEO L, SPECTOR N, DEAR K, LAMBET JM, BLÄTTLER WA, TAYLOR JA, NADLER LM: **Serotherapy of B-cell neoplasms with anti-B4-blocked ricin: a Phase I trial of daily bolus infusion.** *Blood* (1992) 79(3):576-585.

10. GROSSBARD ML, LAMBERT JM, GOLDMACHER VS, BLÄTTLER WA, NADLER LM: **Correlation between *in vivo* toxicity and preclinical *in vitro* parameters for the immunotoxin anti-B4-blocked ricin.** *Cancer Res.* (1992) 52:4200-4207.

11. GROSSBARD ML, LAMBERT JM, GOLDMACHER VS, SPECTOR NL, KINSELLA J, ELISEO L, CORAL F, TAYLOR JA, BLÄTTLER WA, EPSTEIN CL, NADLER LM: **Anti-B4-blocked ricin: a Phase I trial of 7-day continuous infusion in patients with B-cell neoplasms.** *J. Clin. Oncol.* (1993) 11(4):726-737.

12. UCKUN FM, MANIVEL C, ARTHUR D, CHELSTROM LM, FINNEGAN D, TUEL-AHLGREN L, IRVIN JD, MYERS DE, GUNTHER R: ***In vivo* efficacy of B43 (anti-CD19)-pokeweed antiviral protein immunotoxin against human pre-B-cell acute lymphoblastic leukemia in mice with severe combined immunodeficiency.** *Blood* (1992) 79(9):2201-2214.

13. UCKUN FM, CHELSTROM LM, IRVIN JD, FINNEGAN D, GUNTHER R, YOUNG J, KUEBELBECK V, MYERS DE, HOUSTON LL: ***In vivo* efficacy of B43 (anti-CD19)-pokeweed antiviral protein immunotoxin against BCL-1 murine B-cell leukemia.** *Blood* (1992) 79(10):2649-2661.

14. JANSEN B, UCKUN FM, JASZCZ WB, KERSEY JH: **Establishment of a human t(4;11) leukemia in severe combined immunodeficient mice and successful treatment using anti-CD19 (B43)-pokeweed antiviral protein immunotoxin.** *Cancer Res.* (1992) 52:406-412.

15. UCKUN FM: **Immunotoxins for the treatment of leukaemia.** *Br. J. Haematol.* (1993) 85:435-438.

16. ENGERT A, GOTTSTEIN C, WINKLER U, AMLOT P, PILERI S, DIEHL V, THORPE P: **Experimental treatment of human Hodgkin's disease with ricin A-chain immunotoxins.** *Leukemia Lymphoma* (1994) 13(5-6):441-448.

17. WINKLER U, GOTTSTEIN C, SCHÖN G, KAPP U, WOLF J, HANSMANN ML, BOHLEN H, THORPE P, DIEHL V, ENGERT A: **Successful treatment of disseminated human Hodgkin's disease in SCID mice with deglycosylated ricin A-chain immunotoxins.** *Blood* (1994) 83(2):466-475.

18. ENGERT A, GOTTSTEIN C, BOHLEN H, WINKLER U, SCHON G, MANSKE O, SCHNELL R, DIEHL V, THORPE P: **Cocktails of ricin A-chain immunotoxins against different antigens on Hodgkin and Sternberg-Reed cells have superior anti-tumor effects against H-RS cells *in vitro* and solid Hodgkin tumors in mice.** *Int. J. Cancer* (1995) 63(2):304-309.

Ninety percent of mice treated with cocktails containing 2 or 3 ITs had continuous complete remissions as compared with only 40% of mice treated with the same dose of a single IT.

19. SCHNELL R, HATWIG MT, RADSZUHN A, CEBE F, DRILLICH S, SCHÖN G, BOHLEN H, TESCH H, HANSMANN ML, SCHINDLER J, GHETIE V, UHR JW, DIEHL V, VITETTA ES, ENGERT A: **A clinical Phase I study of an anti-CD25-deglycosylated ricin A-chain immunotoxin (RFT5-SMPT-dgA) in patients with refractory Hodgkin's disease.** *Fourth International Symposium on Immunotoxins* (1995). Abstract #161

20. PASQUALUCCI L, WASIK M, TEICHER BA, FLENGHI L, BOLOGNESI A, STIRPE F, POLITO L, FALINI B, KADIN ME: **Antitumor activity of anti-CD30 immunotoxin (Ber-H2/Saporin) *in vitro* and in severe combined immunodeficiency disease mice xenografted with human CD30$^+$ anaplastic large-cell lymphoma.** *Blood* (1995) 85(8):2139-2146.

21. FALINI B, BOLOGNESI A, FLENGHI L, TAZZARI PL, BROE MK, STEIN H, DÜRKOP H, AVERSA F, CORNELI P, PIZZOLO G, BARBABIETOLA G, SABATTINI E, PILERI S, MARTELLI MP, STIRPE F: **Response of refractory Hodgkin's disease to monoclonal anti-CD30 immunotoxin.** *Lancet* (1992) 339:1195-1196.

22. PAI LH, BATRA JK, FITZGERALD DJ, WILLINGHAM MC, PASTAN I: **Anti-tumor activities of immunotoxins made of monoclonal antibody B3 and various forms of *Pseudomonas* exotoxin.** *Proc. Natl. Acad. Sci. USA* (1991) 88:3358-3362.

23. PAI LH, BATRA JK, FITZGERALD DJ, WILLINGHAM MC, PASTAN I: **Antitumor effects of B3-PE and B3-LysPE40 in a nude mouse model of human breast cancer and the evaluation of B3-PE toxicity in monkeys.** *Cancer Res.* (1992) 52:3189-3193.

24. BRINKMANN U, PASTAN I: **Immunotoxins against cancer.** *Biochimica et Biophysica Acta* (1994) 1198:27-45.

25. PAI LH, WITTES RE, SETSER A, GOLDSPIEL B, FITZGERALD D, WILLINGHAM MC, PASTAN I: **Phase I and pharmacokinetic study of the immunotoxin LMB-1 (B3-LysPE38).** *Fourth International Symposium on Immunotoxins* (1995). Abstract #163.

26. WILLINGHAM MC, FITZGERALD DJ, PASTAN I: ***Pseudomonas* exotoxin coupled to a monoclonal antibody against ovarian cancer inhibits the growth of human ovarian cancer cells in a mouse model.** *Proc. Natl. Acad. Sci. USA* (1987) 84:2474-2478.

27. PEARSON JW, FITZGERALD DJP, WILLINGHAM MC, WILTROUT RH, PASTAN I, LONGO DL: **Chemoimmunotoxin therapy against a human colon tumor (HT-29) xenografted into nude mice.** *Cancer Res.* (1989) 49:3562-3567.

28. FITZGERALD D, IDZIOREK T, BATRA JK, WILLINGHAM M, PASTAN I: **Antitumor activity of a thioether-linked immunotoxin: OVB3-PE.** *Bioconjugate Chemistry* (1990) 1:264-268.

29. PAI LH, BOOKMAN MA, OZOLS RF, YOUNG RC, SMITH JW, II, LONGO DL, GOULD B, FRANKEL A, MCCLAY EF, HOWELL S, REED E, WILLINGHAM MC, FITZGERALD DJ, PASTAN I: **Clinical evaluation of intraperitoneal *Pseudomonas* exotoxin immunoconjugate OVB3-PE in patients with ovarian cancer.** *J. Clin. Oncol.* (1991) 9(12):2095-2103.

30. BYERS VS, PIMM MV, SCANNON PJ, PAWLUCZYK I, BALDWIN RW: **Inhibition of growth of human tumor xenografts in athymic mice treated with ricin toxin A chain-monoclonal antibody 791T/36 conjugates.** *Cancer Res.* (1987) 47:5042-5046.

31. BYERS VS, RODVIEN R, GRANT K, DURRANT LG, HUDSON KH, BALDWIN RW, SCANNON PJ: **Phase I study of monoclonal antibody-ricin A chain immunotoxin XomaZyme-791 in patients with metastatic colon cancer.** *Cancer Res.* (1989) 49:6153-6160.

32. BYERS VS, BALDWIN RW: **Targeted kill: from umbrellas to monoclonal antibodies.** *J. Clin. Immunol.* (1992) 12(6):391-405.

33. SPITLER LE: **Clinical studies: solid tumors.** In: *Immunotoxins.* Frankel AE (Ed.), (1988):493-514.

34. SPITLER LE, DEL RIO M, KHENTIGAN A, WEDEL NI, BROPHY NA, MILLER LL, HARKONEN WS, ROSENDORF LL, LEE HM, MISCHAK RP, KAWAHATA RT, STOUDEMIRE JB, FRADKIN LB, BAUTISTA EE, SCANNON PJ: **Therapy of patients with malignant melanoma using a monoclonal antimelanoma antibody-ricin A chain immunotoxin.** *Cancer Res.* (1987) 47:1717-1723.

35. HERTLER AA, SPITLER LE, FRANKEL AE: **Humoral immune response to a ricin A chain immunotoxin in patients with metastatic melanoma.** *Cancer Drug Delivery* (1987) 4(4):245-253.

36. ORATZ R, SPEYER JL, WERNZ JC, HOCHSTER H, MEYERS M, MISCHAK R, SPITLER LE: **Antimelanoma monoclonal antibody-ricin A chain immunoconjugate (XMME-001-RTA) plus cyclophosphamide in the treatment of metastatic malignant melanoma: results of a Phase II trial.** *J. Biol. Response Modifiers* (1990) 9:345-354.

37. MISCHAK RP, FOXALL C, ROSENDORF LL, KNEBEL K, SCANNON PJ, SPITLER LE: **Human antibody responses to components of the monoclonal antimelanoma antibody ricin A chain immunotoxin XomaZyme-MEL.** *Mol. Biol.* (1990) 2:104-109.

38. SELVAGGI K, SARIA EA, SCHWARTZ R, VLOCK DR, ACKERMAN S, WEDEL N, KIRKWOOD JM, JONES H, ERNSTOFF MS: **Phase I/II study of murine monoclonal antibody-ricin A chain (XomaZyme-MEL) immunoconjugate plus cyclosporine A in patients with metastatic melanoma.** *J. Immunother.* (1993) 13:201-207.

39. GHETIE V, VITETTA ES: **Immunotoxins in the therapy of cancer: from bench to clinic.** *Pharmacol. Ther.* (1994) 63:209-234.

40. •• KAMINSKI MS, ZASADNY KR, FRANCIS IR, MILIK AW, ROSS CW, MOON SD, CRAWFORD SM, BURGESS JM, PETRY NA, BUTCHKO GM, GLENN SD, WAHL RL: **Radioimmunotherapy of B-cell lymphoma with [^{131}I]-anti-B1 (anti-CD20) antibody.** *New Engl. J. Med.* (1993) 329(7):459-465.

This paper shows results that support the idea that radioimmunotherapy with [^{131}I]-anti-B1 antibody is a promising new treatment for lymphoma.

41. •• PRESS OW, EARY JF, APPELBAUM FR, MARTIN PJ, NELP WB, GLENN S, FISHER DR, PORTER B, MATTHEWS DC, GOOLEY T, BERNSTEIN ID: **Phase II trial of [^{131}I]-B1 (anti-CD20) antibody therapy with autologous stem cell transplantation for relapsed B-cell lymphomas.** *Lancet* (1995) 346:336-340.

This paper presents the results of a Phase II trial of [^{131}I]-B1 antibody therapy and shows that this therapy associated with autologous stem cell transplantation produces complete responses of long duration in most patients with relapsed B-cell lymphomas.

42. • JUWEID M, SHARKEY RM, MARKOWITZ A, BEHR T, SWAYNE LC, DUNN R, HANSEN HJ, SHEVITZ J, LEUNG S-O, RUBIN AD, HERSKOVIC T, HANLEY D, GOLDENBERG DM: **Treatment of non-Hodgkin's lymphoma with radiolabeled murine, chimeric, or humanized LL2, an anti-CD22 monoclonal antibody.** *Cancer Res.* (1995) 55(Suppl.):5899s-5907s.

This paper shows that an anti-CD22 antibody (LL2) radiolabelled with ^{131}I is a promising agent for the treatment of lymphoma, either with or without autologous bone marrow transplantation.

43. • BUCHSBAUM DJ: **Experimental approaches to increase radiolabeled antibody localization in tumors.** *Cancer Res.* (1995) 55(Suppl.):5729s-5732s.

This review presents the main approaches to improve the localisation of radiolabelled MAbs in tumours: modifying antibodies or radiolabelling techniques, controlling the residence time of radiolabelled MAbs in circulation, modifying the tumour antigen expression and delivery of radiolabelled MAbs, and increasing tumour vascular permeability or blood flow.

44. KAMINSKI MS, FIG LM, ZASADNY KR, KORAL KF, DEL-ROSARIO RB, FRANCIS IR, HANSON CA, NORMOLLE DP, MUDGETT E, LIU CP, MOON S, SCOTT P, MILLER RA, WAHL RL: **Imaging, dosimetry, and radioimmunotherapy with iodine 131-labeled anti-CD37 antibody in B-cell lymphoma.** *J. Clin. Oncol.* (1992) 10(11):1696-1711.

45. • GHETIE M-A, VITETTA ES: **Recent developments in immunotoxin therapy.** *Curr. Opin. Immunol.* (1994) 6:707-714.

This review discusses the improvements and developments in first- and second-generation ITs, as they appear in the papers published in 1993-94.

46. • GHETIE MA, TUCKER K, RICHARDSON J, UHR JW, VITETTA ES: **Eradication of minimal disease in severe combined immunodeficient mice with disseminated Daudi lymphoma using chemotherapy and an immunotoxin cocktail.** *Blood* (1994) 84(3):702-707.

Early treatment of SCID mice bearing human lymphoma with either an IT cocktail or chemotherapy significantly prolonged survival but was not curative. Combination therapy with IT cocktail and chemotherapy cured the mice.

47. YOKOTA S, HARA H, LUO Y, SEON BK: **Synergistic potentiation of *in vivo* antitumor activity of anti-human T-leukemia immunotoxins by recombinant α-interferon and daunorubicin.** *Cancer Res.* (1990) 50:32-37.

48. • JANSEN B, KERSEY JH, JASZCZ WB, GUNTHER R, NGUYEN DP, CHELSTROM LM, TUEL-AHLGREN L, UCKUN FM: **Effective immunochemotherapy of human t(4;11) leukemia in mice with severe combined immunodeficiency (SCID) using B43 (anti-CD19)-pokeweed antiviral protein immunotoxin plus cyclophosphamide.** *Leukemia* (1993) 7(2):290-297.

The results recommend the use of B43-PAP IT plus cyclophosphamide for treatment of refractory or relapsed leukaemias.

49. LOBUGLIO AF, WHEELER RH, TRANG J, HAYNES A, ROGERS K, HARVEY EB, SUN L, GHRAYEB J, KHAZAELI MB: **Mouse/human chimeric monoclonal antibody in man: Kinetics and immune response.** *Proc. Natl. Acad. Sci. USA* (1989) 86:4220-4224.

50. HARRIS WJ, CUNNINGHAM C: In: *Antibody Therapeutics.* RG Landes Company, Austin, TX (1995):85-110

51. REITER Y, PASTAN I: **Antibody engineering of recombinant Fv immunotoxins for improved targeting of cancer: disulfide-stabilized Fv immunotoxins.** *Clin. Cancer Res.* (1996) 2:245-252.

52. ZUCKIER LS, RODRIGUEZ LD, SCHARFF MD: **Immunologic and pharmacologic concepts of monoclonal antibodies.** *Semin. Nuclear Med.* (1989) 29:166-186.

53. PEDLEY RB, BODEN JA, BODEN R, BEGENT RHJ, TURNER A, HAINES AMR, KING DJ: **The potential for enhanced tumour localisation by polyethylene glycol modification of anti-CEA antibody.** *Br. J. Cancer* (1994) 70(6):1126-1130.

54. BATRA JK, KASTURI S, GALLO MG, VOORMAN RL, MAIO SM, CHAUDHARY VK, PASTAN I: **Insertion of constant region domains of human IgG(1) into CD4-PE40 increases its plasma half-life.** *Mol. Immunol.* (1993) 30(4):379-386.

55. KIM JK, TSEN MF, GHETIE V, WARD ES: **Catabolism of the murine IgG1 molecule: evidence that both CH2-CH3 domain interfaces are required for persistence of IgG1 in the circulation of mice.** *Scand. J. Immunol.* (1994) 40:457-465.

56. BUCHSBAUM DJ: **Experimental radioimmunotherapy and methods to increase therapeutic efficacy.** In: *Cancer Therapy with Radiolabeled Antibodies.* Goldberg DM (Ed.), (1994):115-140.

57. MATTES MJ: **On the validity of 'functional affinity' determination for antibodies binding to cell surface antigens or other polyvalent antigens.** *Cancer Res.* (1995) 55(Suppl.):5733s-5735s.

58. • MATTES MJ: **Pharmacokinetics of antibodies and their radiolabels.** In: *Cancer Therapy with Radiolabeled Antibodies.* Goldenberg DM (Ed.), (1994):89-99.

The review discusses the effect of blood clearance and catabolism of radiolabelled MAb and fragments on the penetration of solid tumour by circulating antibody as well as the fate of antibody after binding to cell surface and the role of functional affinity.

59. SUNG C, SHOCKLEY TR, MORRISON PF, DVORAK HF, YARMUSH ML, DEDRICK RL: **Predicted and observed effects of antibody affinity and antigen density on monoclonal antibody uptake in solid tumors.** *Cancer Res.* (1992) 52:377-384.

60. VANHORSSEN PJ, VANOOSTERHOUT YVJM, DEWITTE T, PREIJERS FWMB: **Cytotoxic potency of CD22-ricin A depends on intracellular routing rather than on the number of internalized molecules.** *Scand. J. Immunol.* (1995) 41(6):563-569.

61. YAZDI PT, WENNING LA, MURPHY RM: **Influence of cellular trafficking on protein synthesis inhibition of immunotoxins directed against the transferrin receptor.** *Cancer Res.* (1995) 55:3763-3771.

62. • MAY RD, WHEELER HT, FINKELMAN FD, UHR JW, VITETTA ES: **Intracellular routing rather than cross-linking or rate of internalization determines the potency of immunotoxins directed against different epitopes of sIgD on murine B-cells.** *Cell. Immunol.* (1991) 135:490-500.

The results support the hypothesis that the epitope specificity of a given MAb is an important factor which can determine the intracellular routing of IT after internalisation.

63. BURROWS FJ, WATANABE Y, THORPE PE: **A murine model for antibody-directed targeting of vascular endothelial cells in solid tumors.** *Cancer Res.* (1992) 52:5554-5962.

64. •• BURROWS FJ, THORPE PE: **Eradication of large solid tumors in mice with an immunotoxin directed against tumor vasculature.** *Proc. Natl. Acad. Sci. USA* (1993) 90:8996-9000.

An anti-endothelial cell IT caused remissions in over half of the animals bearing tumours in which an experimental marker was induced on the tumour vascular endothelium.

65. • BURROWS FJ, DERBYSHIRE EJ, TAZZARI PL, AMLOT P, GAZDAR AF, KING SW, LETARTE M, VITETTA ES, THORPE PE: **Up-regulation of endoglin on vascular endothelial cells in human solid tumors: implications for diagnosis and therapy.** *Clin. Cancer Res.* (1995) 1:1623-1634.

The findings suggest that an antibody that recognises endoglin might have therapeutic value for the treatment of solid tumours in humans by selectively killing dividing endothelial cells which are prevalent in such tumours.

66. THORPE P, HUANG X, DERBYSHIRE E, KING S, MOLEMA G, EDGINGTON T: **Tumor infarction by immunoconjugates that coagulate the vasculature of solid tumors.** *Fourth International Symposium on Immunotoxins* (1995). Abstract #39.

67. GOLDMACHER VS, LAMBERT JM, BLATTLER WA: **The specific cytotoxicity of immunoconjugates containing blocked ricin is dependent on the residual binding capacity of blocked ricin: evidence that the membrane binding and A-chain translocation activities of ricin cannot be separated.** *Biochem. Biophys. Res. Commun.* (1992) 183(2):758-766.

68. LAMBERT JM, GOLDMACHER VS, COLLINSON AR, NADLER LM, BLÄTTLER WA: **An immunotoxin prepared with blocked ricin: a natural plant toxin adapted for therapeutic use.** *Cancer Res.* (1991) 51:6236-6242.

69. GROSSBARD ML, NADLER LM: **Monoclonal antibody therapy for indolent lymphomas.** *Semin. Oncol.* (1993) 20(Suppl. 5):118-135.

70. WESTON S, TUCKER A, THATCHER D, DERBYSHIRE D, PAUPTIT R: **X-ray structure of recombinant ricin A-chain at 1.8 Å resolution.** *J. Mol. Biol.* (1994) 244:410-422.

71. •• CHIGNOLA R, ANSELMI C, SERRA MD, FRANCESCHI A, FRACASSO G, PASTI M, CHIESA E, LORD JM, TRIDENTE G, COLOMBATTI M: **Self-potentiation of ligand-toxin conjugates containing ricin A chain fused with viral structures.** *J. Biol. Chem.* (1995) 270(40):23345-23351.

The results have shown the ability of vesicular stomatitis virus peptide to improve the translocation of ricin A chain to the target cell cytosol, thereby improving its specific cytotoxicity by more than a hundred times.

72. GHETIE V, SWINDELL E, UHR JW, VITETTA ES: **Purification and properties of immunotoxins containing one *vs.* two deglycosylated ricin A chains.** *J. Immunol. Methods* (1993) 166:117-122.

73. GHETIE V, ENGERT A, SCHNELL R, VITETTA ES: **The *in vivo* anti-tumor activity of immunotoxins containing two *vs.* one deglycosylated ricin A chains.** *Cancer Lett.* (1996) 98:97-101.

74. DHANABAL M, FRYXELL DK, RAMAKRISHNAN S: **A novel method to purify immunotoxins from free antibodies using modified recombinant toxins.** *J. Immunol. Methods* (1995) 182:165-177.

75. FLAVELL DJ, BOEHM DA, EMERY L, NOSS A, FLAVELL SU: **Comparison of the therapeutic efficacy of anti-CD7-saporin immunotoxins constructed with one or two saporin moieties per immunotoxin molecule in SCID mice with human T-cell acute leukemia.** *Fourth International Symposium on Immunotoxins* (1995) Abstract #135.

76. WEBBER KO, REITER Y, BRINKMANN U, KREITMAN R, PASTAN I: **Preparation and characterization of a disulfide-stabilized Fv fragment of the anti-tac antibody: comparison with its single-chain analog.** *Mol. Immunol.* (1995) 32(4):249-258.

77. •• REITER Y, PAI L, BRINKMANN U, WANG QC, PASTAN I: **Antitumor activity and pharmacokinetics in mice of a recombinant immunotoxin containing a disulfide-stabilized Fv fragment.** *Cancer Res.* (1994) 54:2714-2718.

Disulphide-stabilised Fv fragment of an antibody fused to PE38KDEL is more stable and can be produced with improved yield compared with Fv fused by using a peptide linker. This IT caused complete regression of human tumours grafted in SCID mice with no toxic effect on the animals.

78. WALES R, CHADDOCK JA, ROBERTS LM, LORD JM: **Addition of an ER retention signal to the ricin A-chain increases the cytotoxicity of the holotoxin.** *Exp. Cell Res.* (1992) 203:1-4.

79. FERNANDEZ-BOTRAN R, YANG C, THORPE P: **Modification of ricin A chain-containing immunotoxins with the endoplasmic reticulum retrieval consensus sequence (KDEL).** *Third International Symposium on Immunotoxins* (1992). Abstract:17.

80. ANDERSON DC, MANGER R, SCHROEDER J, WOODLE D, BARRY M, MORGAN A, FRITZBERG A: **Enhanced *in vitro* tumor cell retention and internalization of antibody**

derivatized with synthetic peptides. *Bioconjugate Chemistry* (1993) 4:10-18.

81. SOLER-RODRIGUEZ A-M, GHETIE M-A, OPPENHEIMER-MARKS N, UHR JW, VITETTA ES: **Ricin A-chain and ricin A-chain immunotoxins rapidly damage human endothelial cells: implications for vascular leak syndrome.** *Exp. Cell Res.* (1993) 206:227-234.

82. BALUNA R, GHETIE V, OPPENHEIMER-MARKS N, VITETTA ES: **The binding of ricin A-chain to fibronectin: possible implications for vascular leak syndrome in immunotoxin-treated patients.** *Submitted for publication.*

83. KUAN C-T, PAI L, PASTAN I: **Immunotoxins containing *Pseudomonas* exotoxin that target LeY damage human endothelial cells in an antibody-specific mode: relevance to vascular leak syndrome.** *Clin. Cancer Res.* (1995) 1:1589-1594.

84. SIEGALL C, LIGGITT D, CHACE D, TEPPER M, FELL HP: **Prevention of immunotoxin-mediated vascular leak syndrome in rats with retention of antitumor activity.** *Proc. Natl. Acad. Sci. USA* (1994) 91:9514-9518.

85. CHOYNICK A, SCHNEIDER W, TSO JY, QUEEN C, CHANG CM: **A recombinant, membrane-acting immunotoxin.** *Cancer Res.* (1991) 51:465-467.

86. • WALKER BJ, KRISHNASASTRY M, ZORN L, BAYLEY H: **Assembly of the oligomeric membrane pore formed by staphylococcal α-hemolysin examined by truncation mutagenesis.** *J. Biol. Chem.* (1992) 267:21782-21786.

This review presents various pore-forming proteins that have potential value in drug delivery as components of ITs that can be activated at the surface of target cells.

87. • GADINA M, NEWTON DL, RYBAK SM, WU Y-N, YOULE RJ: **Humanized immunotoxins.** *Therapeutic Immunology* (1994) 1:59-64.

This review presents the use of human protein in place of plant and bacterial proteins to generate fully human ITs which may be better tolerated and less antigenic than the currently used ITs.

88. BAYLEY H: **Triggers and switches in a self-assembling pore-forming protein.** *J. Cell Biochem.* (1994) 56(2):177-182.

89. SENTER PD, WALLACE PM, SVENSSON HQ, VRUDHULA V, KERR DE, HELLSTRÖM I, HELLSTRÖM KE: **Generation of cytotoxic agents by targeted enzymes.** *Bioconjugate Chemistry* (1993) 4:3-9.

90. RODRIGUES ML, PRESTA LG, KOTTS CE, WIRTH C, MORDENTI J, OSAKA G, WONG WLT, NUIJENS A, BLACKBURN B, CARTER P: **Development of a humanized disulfide-stabilized anti-beta p185 (HER2) Fv-beta-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug.** *Cancer Res.* (1995) 55(1):63-70.

91. SVENSSON HP, VRUDHULA V, EMSWILER J, MACMASTER J, COSAND W, SENTER P, WALLACE P: ***In vitro* and *in vivo* activities of a doxorubicin prodrug in combination with monoclonal antibody β-lactamase conjugates.** *Cancer Res.* (1995) 55:2357-2365.

92. • KERR D, SCHREIBER G, VRUDHULA V, SVENSSON H, HELLSTRÖM I, HELLSTRÖM K, SENTER P: **Regressions and cures of melanoma xenografts following treatment with monoclonal antibody β-lactamase conjugates in combination with anticancer prodrugs.** *Cancer Res.* (1995) 55:3558-3563.

Experiments in mice with established tumours have shown that cures were obtained in 80% of the animals treated with prodrug and antibody beta-lactamase conjugate.

93. DOHLSTEN M, ABRAHMSÉN L, BJÖRK P, LANDO P, HEDLUND G, FORSBERG G, BRODIN T, GASCOIGNE N, FORBERG C, LIND P, KALLAND T: **Monoclonal antibody-superantigen fusion proteins: tumor-specific agents for T-cell-based tumor therapy.** *Proc. Natl. Acad. Sci. USA* (1994) 91:8945-8949.

94. DOHLSTEN M, LANDO P, BJÖRK P, ABRAHMSÉN L, OHLSSON L, LIND P, KALLAND T: **Immunotherapy of human colon cancer by antibody-targeted superantigens.** *Cancer Immunol. Immunother.* (1995) 41:162-168.

95. • LANDO P, DOHLSTEN M, OHLSSON L, KALLAND T: **Tumor-reactive superantigens suppress tumor growth in humanized SCID mice.** *Int. J. Cancer* (1995) 62:466-471.

The antitumour effect of Fab-SEA antibody was demonstrated in lymphocyte-engrafted humanised SCID mice bearing human colon carcinomas.

96. SCHERER MT, IGNATOWICZ L, WINSLOW G, KAPPLER J, MARRACK P: **Superantigens: bacterial and viral proteins that manipulate the immune system.** *Ann. Rev. Cell Biol.* (1993) 9:101-128.

97. OCHI A, MIGITA K, XU J, SIMINOVITCH K: ***In vivo* tumor immunotherapy by a bacterial superantigen.** *J. Immunol.* (1993) 151(6):3180-3186.

98. KRULL F, HOLZER U, IHLE J, BETHGE W, DOHLSTEN M, KALLAND T, FIERLBECK G, HANDGRETINGER R, REISFELD R, NIETHAMMER D, DANNECKER GE: **Antibody targeted lysis of melanoma cells by superantigen activated T-cells.** *The Ninth International Congress of Immunology* (1995). Abstract #5227.

99. GRAZIANO RF, SOMASUNDARAN C, GOLDSTEIN J: **The production of bispecific antibodies.** Fanger MW (Ed.), RG Landes Co., Austin (1995):1-26.

100. SEGAL DM, BAKACS T, JOST CR, KURUCZ I, SCONOCCHIA G, TITUS J: **T-cell-targeted cytotoxicity.** Fanger MW (Ed.), RG Landes Co., Austin (1995):27-41.

101. WALLACE PK, VALONE FH, FANGER MW: **Myeloid cell-targeted cytotoxicity of tumor cells.** Fanger MW (Ed.), RG Landes Co., Austin (1995):43-76.

102. • HAYDEN MS, LINSLEY PS, GAYLE MA *et al.*: **Single chain mono- and bispecific antibody derivatives with novel biological properties and anti-tumor activity from a COS cell transient expression system.** *Ther. Immunol.* (1994) 1:3-15.

A new bispecific antibody family is described consisting of single chain bispecific antibody fused with CH_2-CH_3 domain of IgG.

103. GRIFFITHS GL: **Radiochemistry of therapeutic radionuclides.** In: *Cancer Therapy with Radiolabeled Antibodies.* Goldenberg DM (Ed.), (1994):47-62.

104. JURCIC JG, SCHEINBERG DA: **Recent developments in the radioimmunotherapy of cancer.** *Curr. Opin. Immunol.* (1994) 6:715-721.

105. FOXWELL B, BAND H, LONG J, JEFFERY W, SNOOK D, THORPE P, WATSON G, PARKER P, EPENETOS A, CREIGHTON A: **Conjugation of monoclonal antibodies to a synthetic peptide substrate for protein kinase: a method for labeling antibodies with ^{32}P.** *Br. J. Cancer* (1988) 57:489-493.

106. LEUNG S-O, KARACAY H, LOSMAN M, GRIFFITHS G, GOLDENBERG D, HANSEN H: **Bacterial expression of a kemptide fusion protein facilitates ^{32}P labeling of a humanized, anti-carcinoembryonic antigen (hMN-14) antibody fragment.** *Cancer Res.* (1995) 55(Suppl.):5968s-5972s.

107. PIETERSZ GA: **The linkage of cytotoxic drugs to monoclonal antibodies for the treatment of cancer.** *Bioconjugate Chemistry* (1990) 1:89-95.

108. CHARI RVJ, MARTELL BA, GROSS JL, COOK SB, SHAH SA, BLÄTTLER WA, MCKENZIE S, GOLDMACHER V: **Immunoconjugates containing novel maytansinoids: promising anticancer drugs.** *Cancer Res.* (1992) 52:127-131.

109. HINMAN LM, HAMANN PR, WALLACE R, MENENDEZ AT, DURR FE, UPESLACIS J: **Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics.** *Cancer Res.* (1993) 53:3336-3342.

110. TRAIL PA, WILLNER D, LASCH SJ, HENDERSON AJ, HOFSTEAD S, CASAZZA AM, FIRESTONE RA, HELLSTRÖM I, HELLSTRÖM KE: **Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates.** *Science* (1993) 261:212-215.

111. SCHOLAR EM, TOEWS ML: **Inhibition of invasion of murine mammary carcinoma cells by the tyrosine kinase inhibitor genistein.** *Cancer Lett.* (1994) 87(2):159-162.

112. UCKUN FM, EVANS WE, FORSYTH CJ, WADDICK KG, TUEL-AHLGREN L, CHELSTROM LM, BURKHARDT A, BOLEN J, MYERS DE: **Biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinases.** *Science* (1995) 267:886-891.

113. MYERS DE, JUN X, WADDICK KG, FORSYTH C, CHELSTROM LM, GUNTHER RL, TUMER N, BOLEN J, UCKUN FM: **Membrane-associated CD19-LYN complex is an endogenous p53-independent and Bcl-2-independent regulator of apoptosis in human B-lineage lymphoma cells.** *Proc. Natl. Acad. Sci. USA* (1995) 92:9575-9579.

114. WEIL-HILLMAN G, UCKUN FM, MANSKE JM, VALLERA DA: **Combined immunochemotherapy of human solid tumors in nude mice.** *Cancer Res.* (1987) 47:579-585.

115. UCKUN FM, CHELSTROM LM, FINNEGAN D, TUEL-AHLGREN L, MANIVEL C, IRVIN JD, MYERS DE, GUNTHER R: **Effective immunochemotherapy of CALLA$^+$Cμ$^+$ human pre-B acute lymphoblastic leukemia in mice with severe combined immunodeficiency using B43 (anti-CD19) pokeweed antiviral protein immunotoxin plus cyclophosphamide.** *Blood* (1992) 79(12):3116-3129.

116. GHETIE M-A, PODAR EM, GORDON BE, PANTAZIS P, UHR JW, VITETTA ES: **Combination immunotoxin treatment and chemotherapy in SCID mice with advanced, disseminated Daudi lymphoma.** *Submitted for publication.*

Maria-Ana Ghetie, Victor Ghetie & Ellen S Vitetta, Department of Microbiology and Cancer Immunobiology Center, University of Texas Southwestern Medical Center, 6000 Harry Hines, Dallas, Texas 75235, USA.

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(71) Applicant: BRISTOL-MYERS SQIBB COMPANY [US/US]; 345 Park Avenue, New York, NY 10154 (US).

(72) Inventors: WOLFF, Edith, Ann ; 5451 42nd Avenue SW, Seattle, WA 98136 (US). RAFF, Howard, V. ; 2552 11th Avenue West, Seattle, WA 98119 (US).

(74) Agent: POOR, Brian, W.: Bristol-Myers Squibb Company, 3005 First Avenue, Seattle, WA 98121 (US).

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(54) Title: HOMOCONJUGATED IMMUNOGLOBULINS

(57) Abstract

Homoconjugated antibodies with high avidity for antigen have increased therapeutic activies and are utilized in pharmaceutical and diagnostic compositions. The homoconjugates, typically prepared from monoclonal antibodies of the IgG class which bind to the same antigenic determinant, are covalently linked by synthetic cross-linking. The homoconjugates are comprised of at least two immunoglobulin monomers so as to provide an IgG-like molecule which is tetravalent, hexavalent or more for the selected antigen. The homoconjugates are able to cross the placenta. Methods of treatment using these homoconjugates are also provided.

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HOMOCONJUGATED IMMUNOGLOBULINS

Related Application

The present application is a continuation-in-part of Ser. No. 07/575,725, filed August 31, 1990.

Background Of The Invention

Monoclonal antibodies offer great promise as exquisitely specific immunotherapeutic agents with potentially minimal side effects. Thus, monoclonal antibodies are being developed for a wide variety of applications, such as the treatment of tumors, infectious diseases and autoimmune disorders, regulation of the immune system, and others. Unfortunately, few monoclonal antibodies have the qualities that enable them to successfully make the transition from research and development to clinical regimen.

The therapeutic or diagnostic usefulness of a monoclonal antibody results from several factors, in addition to simply binding the desired antigen. The antibody must possess sufficient binding affinity, a measure of the inherent strength of the antibody binding to its corresponding epitope. It must also have a relatively high level of avidity, which reflects the overall stability of the antibody-antigen complex and is based on the valency of the antibody (and antigen) and the geometric arrangement of the interacting components. The affinity and avidity of different antibodies can vary widely.

Often the monoclonal antibody which is selected must be of an appropriate isotype or subclass thereof to efficiently initiate desired effector functions. These functions include fixation of complement, binding to effector macrophages or polymorphonuclear leukocytes, or other properties that may be required in a particular therapeutic application. Isotype also affects antibody bio-distribution, half-life, transplacental passage, and other characteristics.

In general, IgG antibodies would be preferred over IgM antibodies for most therapeutic uses. When compared to IgMs, IgGs typically possess longer in vivo half-lives, are able to cross the placenta to the fetus, and when formulated as a pharmaceutical composition may have a longer shelf life. IgG molecules are monomeric, however, and have only two antigen binding sites so the avidity is much lower than with a comparable IgM antibody, which is pentavalent and has ten antigen binding sites.

With conventional technology it is frequently very difficult to identify monoclonal antibodies having the desired antigen binding specificity, affinity, avidity and effector functions. Recombinant DNA techniques have been developed to avoid the unpredictable and labor intensive method of simply screening large numbers of antibody-producing fused or transformed cells. Genes encoding the antigen binding variable (or hypervariable) regions of an antibody having a desired binding specificity have been cloned next to genes encoding antibody constant regions which mediate desired effector functions. See, for example, U.S. Pat. 4,816,397, European Patent Office publications EP 173,494 and 239,400 and PCT publication WO 89/07142. Such procedures can also be quite laborious and have had only limited experimental validation. Even with these procedures one may still be faced with a recombinant IgG antibody not having sufficient avidity to initiate

biologically important effector functions, or with IgM molecules which have a desired therapeutic activity but suffer from the general disadvantages associated with IgMs as mentioned above.

The avidity of IgG antibodies could be improved by increasing the valency of the molecule to greater than two. More interactions between antibody and antigen would result in tighter binding and would stabilize the antibody-antigen interaction, generally an important attribute for therapeutic use. IgG antibodies of high avidity (via multivalent attachment) and which have the desired effector functions would be greatly preferred over comparable antibodies of low avidity, but to date antibodies having these characteristics have not been described.

Accordingly, what is needed in the art is a means for producing high avidity IgG antibodies having desired effector functions while avoiding many of the difficulties inherent in working with IgMs. Quite remarkably, the present invention fulfills this and other related needs.

Summary of the Invention

Homoconjugated antibodies possess increased therapeutic effectiveness when compared to the corresponding parental antibody monomer. This activity may be due to, inter alia, interactions of higher avidity and increased effector functions. Accordingly, antibodies which bind to the same antigen, and more particularly to the same antigenic determinant, are covalently bonded via cross-linking to one another by synthetic chemical coupling to produce such homoconjugates. Generally, the homoconjugates comprise

at least two to three antibody molecules, typically of the IgG class. The antibodies are preferably monoclonal antibodies, and may be any of a variety of species. For administration to humans the antibodies will usually be human or murine in origin or have human constant regions.

Accordingly, pharmaceutical compositions are provided which comprise a pharmaceutically acceptable carrier and at least two IgG antibody molecules, which bind to substantially the same antigenic determinant, chemically bonded to one another by synthetic covalent linkage. The homoconjugated antibodies and pharmaceutical compositions thereof can be used therapeutically in methods of treatment of antigen related diseases to, e.g., protect against infection, such as by E. coli or group B streptococci, inhibit the growth of tumors, including breast and other tumors, regulate the immune response, and the like. As homoconjugates of IgG antibodies are able to pass the placenta the preparations can be used to treat a fetus in utero.

In another related aspect the invention provides a substantial improvement in methods for therapeutic administration of monoclonal antibodies to a patient for treatment of an antigen related disease. The improvement comprises administering to the patient covalently cross-linked homoconjugated monoclonal antibodies having at least two IgG antibody molecules which bind to the same antigenic determinant of the antigen related to the disease. In preferred embodiments the antibodies are cross-linked via disulfide bonds.

Brief Description of the Figures

Fig. 1 shows chromatograms of FPLC profiles of the IgG homoconjugate mixtures, with retention time along x-axis and A_{280} along y-axis; Peaks labeled A, B and C, represent trimer, dimer and monomer fractions, respectively;

Fig. 2 illustrates the increased binding activity in EIAs of homoconjugates (dimers or trimers) compared to initial monomers of monoclonal antibody D3, a human IgG monoclonal antibody which binds to the group carbohydrate of group B streptococci;

Fig. 3 illustrates the increased binding activity of homoconjugates (dimers or trimers) compared to initial monomers of monoclonal antibody 5E1-G, a human IgG monoclonal antibody which binds to the capsular carbohydrate of E. coli K1;

Fig. 4 illustrates the increased binding activity in EIAs of homoconjugates (dimers) compared to initial monomers of BR64, a murine IgG monoclonal antibody which binds to a human breast tumor associated antigen;

Fig. 5 illustrates the comparative binding activity of homoconjugated chimeric BR96 antibody against tumor cell lines, where Fig. 5A shows binding activity against human breast tumor cell line H3760B, Fig. 5B shows binding activity against human lung tumor cell line H2707, Fig. 5C shows binding activity against human lung tumor cell line H2987, and Fig. 5D is binding activity against human breast tumor cell line H3396;

Fig. 6 shows the increased opsonic activity against group B streptococci by dimer and trimer homoconjugates of human monoclonal antibody D3 compared to the initial IgG monomer;

Fig. 7 shows the enhanced opsonophagocytosis by monoclonal antibody D3 homoconjugates against group B

streptococcal strains M94 and I334 compared to the activity by the D3 monomer antibody;

Fig. 8 shows the increased opsonic activity against E. coli K1 of dimer and trimer homoconjugates of human monoclonal antibody 5E1-G compared to the initial IgG monomer;

Fig. 9 depicts enhanced opsonophagocytosis conferred by homoconjugates of monoclonal antibody 5E1-G against strains H16 and A14 of E. coli K1 compared to the antibody monomer;

Fig. 10 shows increased complement dependent cytotoxicity against breast tumor cell line H3630 by dimer homoconjugates of monoclonal antibody BR64 compared to the initial IgG monomer;

Fig. 11 illustrates the cytotoxicity shown by BR96 homoconjugates and monomeric monoclonal antibody against breast tumor cell line H3396; and

Fig. 12 shows the in vivo protection conferred by homoconjugates of monoclonal antibody D3 and control monomer at different concentrations of antibody.

Description of the Specific Embodiments

The present invention provides homoconjugates of monoclonal antibodies against selected antigens, and methods for preparing such homoconjugates. By chemically linking antibody molecules, homoconjugates are prepared which possess increased valency and two or more Fc regions. By this means a variety of effects may be accomplished, including, inter alia, increases in binding avidity, complement fixation, cellular activation, opsonophagocytosis, etc. Thus the invention provides the ability to convert antibodies of perhaps limited in vivo utility to antibodies having characteristics significantly more conducive to a desired therapeutic

activity. For example, homoconjugation may serve to convert an IgG monomer of low binding avidity to one of higher avidity and better able to promote effector functions that were perhaps not previously attainable.

By homoconjugate is meant the covalent association or linking of two, three or more antibody molecules which bind to the same antigenic determinant, thereby forming antibody homodimers, homotrimers, etc. The homoconjugates may be prepared from two, three or more different monoclonal antibodies (i.e., those produced by different immortalized cell lines) which bind to the same antigenic determinants (epitopes) on the antigen. The monoclonal antibodies which comprise the homoconjugate may be different (produced by distinct cell lines) but preferably they are the same, i.e., obtained from the same cell line, and thus constitute a relatively homogeneous preparation of monoclonal antibodies with virtually identical antigen binding specificity. By binding to the same or substantially the same epitope is meant to refer to monoclonal antibodies which are capable of reciprocal or non-reciprocal competition with the other for binding to the antigen. One skilled in the art will know how to conduct competition immunoassays, such as by radioimmunoassay or enzyme immunoassay, as generally described in, e.g., U.S. Pat. 3,817,837; Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1988); and Day, Advanced Immunochemistry, 2d ed., Wiley-Liss Publications, NY (1990), each incorporated herein by reference.

The Fc regions of the monoclonals used for homoconjugation, or other aspects of the immunoglobulin molecule which do not substantially affect antigen binding specificity, may also be altered to produce desired effector functions. For example, it may be desirable to substitute a Fc domain for protein A binding into a molecule not having that capacity, for ease of

purification or the like. Other substitutions may provide for decreased immunogenicity, increased or decreased complement activation, cell receptor binding, control of catabolic rate, placental and gut transfer, ability to participate in antibody-dependent cellular cytotoxicity, and other aspects of immune regulation. A number of antibody functions have been localized to a constant region domain or domains. See, Paul, Fundamental Immunology, Raven Press, New York, NY, 1984, incorporated herein by reference. A wide variety of techniques are available to produce recombinant immunoglobulins, e.g., U.S. Pat. 4,816,397, European Patent Office publications EP 173,494 and 239,400 and PCT publication WO 89/07142, each incorporated herein by reference. Accordingly, the homoconjugated immunoglobulins may be any of the heavy chains and subclasses thereof. The light chains may be either kappa or lambda.

Particularly preferred in the present invention are homoconjugates of antibodies having gamma heavy chains, so as to form homoconjugated multivalent IgG molecules. Within the IgG subclasses of 1, 2, 3 and 4 (human) and 1, 2a, 2b and 3 (murine), human subclasses 1 and 3 and murine subclasses 1, 2a and 2b are generally preferred for applications requiring maximum complement fixation, binding to monocytes, macrophages and polymorphonuclear cells, and the ability to cross the placenta. The effector functions of human IgG_2 and IgG_4 antibodies may also be substantially increased by the homoconjugation procedures described herein.

It is also contemplated that under certain circumstances, depending on the intended use, antibodies having alpha, mu, epsilon or delta type heavy chains may also be employed for homoconjugation as described herein.

The binding affinity of the antibodies for use in homoconjugates will vary, but will generally be at least 10^{-4} M, typically at least about 10^{-6} M to 10^{-7} M,

and preferably at least about 10^{-8} to 10^{-9} M or greater. The avidity of the homoconjugates prepared from such antibodies should generally be at least about 10^{-6} M to 10^{-7} M, and preferably at least about 10^{-8} to 10^{-10} M or greater. Means for determining affinity and avidity are known, as described in Day, Advanced Immunochemistry, supra. While the homoconjugates may have quantitative increases in avidity, generally the homoconjugates should also have qualitative increases in avidity and effector functions, e.g., those evidenced by antigen binding tests and other functional assays as described herein and as will generally be known to one of ordinary skill in the art.

The homoconjugated immunoglobulins may be of any species or combination thereof from which monoclonal antibodies may be prepared. Although it has generally been relatively easy to produce murine monoclonal antibodies of a desired antigen binding specificity, it has been much more difficult to produce human monoclonal antibodies of the desired specificity and having the desired constant region properties. Human monoclonal antibodies are preferable for many applications, especially in vivo diagnosis and therapy of humans to minimize their recognition as foreign by a patient's immune system.

While murine and human immunoglobulins are most commonly produced, monoclonal antibodies or portions thereof originating with other species, such as lagomorpha, bovine, ovine, equine, porcine, avian or the like may be employed. It should be understood that the monoclonal antibody art and genetic engineering techniques have advanced sufficiently such that antibody sequences of one species may be interchanged with those of another species. Thus, as used herein, a "human" antibody, for example, refers to one that is substantially human in origin but may also contain some non-human and/or non-immunoglobulin sequences.

Similarly, when referring to immunoglobulin, used synonymously herein with antibody, it will be understood that some non-immunoglobulin sequences may be present in the molecule while retaining the ability to bind antigen. Immunoglobulin refers to both whole immunoglobulins and binding fragments thereof.

The antibodies which are used for homoconjugation may be substantially monospecific, i.e., relatively pure preparations of substantially homogeneous antibodies obtained from polyclonal antisera, or may be monoclonal antibodies. Monoclonal antibodies which bind to a desired antigen or epitope thereof are obtained from an established cell line which secretes them. The antibody-producing cell line may be isolated from B cells of several species using conventional fusion, viral transformation or other immortalization techniques well known to those skilled in the art. For instance, human monoclonal antibodies may be generated using Epstein-Barr virus (EBV) transformation, hybridoma fusion techniques, or combinations thereof. See, for example, Kozbor et al., Proc. Natl. Acad. Sci. USA 79:6651 (1982), and U.S. Pat. Nos. 4,464,465 and 4,624,921, which are incorporated herein by reference. By monoclonal antibody is meant an antibody produced by a clonal, immortalized cell line separate from cells producing antibodies with a different antigen binding specificity. Thus such monoclonal antibodies are produced and isolated from other monoclonal antibodies and, accordingly, in substantially pure form (relative to other antibodies) and at a concentration generally greater than normally occurring in sera from the animal species which serves as the B cell source.

Thus, it should be understood that the invention is not limited by the antigen binding specificity of the particular homoconjugates exemplified herein, but rather, it can be used in the treatment of a variety of antigen related diseases, particularly those

for which monoclonal antibodies have been therapeutically administered. By antigen related disease is meant a disease whose manifestation coincide with the clinical presence of a foreign antigen (e.g., bacteria, virus, tumor or tumor associated antigen) or self antigen (as with autoimmune diseases). A wide variety of monoclonal antibodies have been described in the technical and patent literature, many of which are publicly available from cell depositories, such as the American Type Culture Collection, 12301 Parklawn Dr., Parkville, MD 20852, whose catalogue, ATCC Catalogue of Cell Lines and Hybridomas, 6th ed. (1988), is incorporated herein by reference. Representative examples of monoclonal antibodies are described in, e.g., U.S. Pat. Nos. 4,596,769, 4,689,299, 4,753,894, 4,834,975, 4,834,976, 4,925,800, and 4,958,009, each of which is incorporated herein by reference. The methods described herein provide the ability to produce novel cross-linked homoconjugates from immunoglobulins obtained from such cell lines.

The chemically linked homoconjugated immunoglobulins will be produced by chemical conjugation of antibodies using well known laboratory procedures, such as by employing cross-linking reagents. By chemically linked is meant that the immunoglobulin molecules are synthetically linked, i.e., not produced as such by a cell, to one another by covalent bonds. A preferred method of conjugation is the formation of at least one covalent bond between the immunoglobulin molecules.

The immunoglobulin molecules are complexed or chemically bonded together by any of a variety of well known chemical linking procedures. The Fc regions or Fab regions may serve as the site of the linkage. The linkage may be direct, which includes linkages containing a synthetic linking group, or indirect, by which is meant a link having an intervening moiety, such as a protein or

peptide, e.g., plasma albumin, or other spacer molecule. For example, the linkage may be by way of heterobifunctional or homobifunctional cross-linkers, e.g., carbodiimide, glutaraldehyde, N-succinimidyl 3-(2-pyridydithio) propionate (SPDP) and derivatives, bis-maleimide, 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), cross-linking without exogenous cross-linkers by means of groups reactive with the individual molecules, such as carbohydrate, disulfide, carboxyl or amino groups via oxidation or reduction of the native protein, or treatment with an enzyme or the like. Methods for chemically cross-linking antibody molecules are generally known in the art, and a number of hetero- and homobifunctional agents are described in, e.g., U.S. Pat. Nos. 4,355,023, 4,657,853, 4,676,980, 4,925,921, and 4,970,156, and in Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Press, 1988, Cold Spring Harbor, NY and ImmunoTechnology Catalogue and Handbook, Pierce Chemical Co. (1989), each of which patents and publications is incorporated herein by reference. In general, such synthetic cross-linking should not substantially affect the antigen binding region of the molecules nor the desired effector functions.

Detection and purification of the homoconjugated immunoglobulins may be accomplished by a variety of techniques, including liquid and affinity chromatography, gradient centrifugation, and gel electrophoresis, among others. Increased activity of the homoconjugates may be measured by quantitative antigen binding assays, antibody competition experiments, opsonophagocytic assays, complement dependent cytotoxicity assays, and the like. These techniques are familiar to those skilled in the art, and are described in, for example, Harlow and Lane, supra.

Homoconjugated antibody preparations with increased binding ability will likely be useful in the

treatment and diagnosis of a wide variety of conditions referred to herein as antigen related diseases. The homoconjugates will offer significantly improved therapeutic and diagnostic characteristics compared to the unconjugated monomeric antibody. Due to the increased avidity of the homoconjugates, it is now possible in certain instances to convert a previously non-protective or weakly protective IgG antibody to be protective against infection or tumors, for example, or to act as an immunomodulator by potentiating or otherwise regulating a host's immune response to a particular antigen. Where an IgM antibody to an antigen or particular epitope of the antigen is protective and a monomeric IgG antibody is non-protective or weakly protective, a homoconjugate produced using the methods described herein may provide sufficient avidity to confer significant protection against infection, cell killing, etc. For instance, an IgG dimer or trimer homoconjugate may possess therapeutic anti-infective qualities that may be found with certain multivalent antibodies such as IgMs, but also have qualities inherent to IgG monomers, such as their ability to cross the placenta, to bind to macrophages and PMNs, and the lack of a requirement for complement to mediate opsonization. The IgG homoconjugates may possess other attributes typically associated with IgGs, such as ease of purification, increased stability, increased shelf life, and increased half-life in vivo.

Although the homoconjugate preparations will be useful against a range of targets, such as bacterial and viral antigens, depending of course on the particular specificity of a homoconjugate's antigen binding region, they will be especially useful where the killing of mammalian cells is required. For example, the homoconjugates can be used for the treatment of cancer cells which display particular tumor-associated antigens (e.g., breast or lung tumor associated antigens), the

inhibition or killing of mammalian cells infected with viruses or bacteria or cells which express antigens associated with a particular autoimmune disease. The homoconjugates can also be used to eliminate selected cells from bone marrow or in the immunosuppression of graft recipients, etc.

Of course, it is understood that the present invention is not limited to antibody homoconjugates which are protective or show other such functional attributes in vivo, as increased avidity also makes feasible an array of diagnostic procedures perhaps not otherwise available to a bivalent monomer of low affinity and/or low avidity.

The ability of the resultant antibodies to inhibit a tumor, such as a breast or lung tumor, to act as an immunomodulator, or to protect against challenge by a pathogen, for example, can be measured in a wide variety of in vitro and in vivo systems, as will be known to the skilled artisan. An exemplary protocol for protection against E. coli K1, using a homoconjugated antibody which was non-protective or weakly protective as an IgG, appears in Example III below.

The novel homoconjugates of monoclonal antibodies and pharmaceutical compositions prepared therefrom are particularly useful for administration for prophylactic and/or therapeutic treatment of an antigen-related disease. Preferably, the pharmaceutical compositions can be administered parenterally, i.e., subcutaneously, intramuscularly or intravenously, or orally. Thus, this invention provides compositions for parenteral administration which comprise a solution of the homoconjugated monoclonal antibody preparations or a cocktail of homoconjugated and monomeric antibodies dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These compositions may be sterilized by

conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected, the condition being treated, e.g., an infectious disease such as a group B streptococcal or E. coli infection, a tumor, such as breast carcinoma, etc., and the subject being treated, i.e., an adult, child or neonate.

Thus, a typical pharmaceutical composition for intravenous infusion to treat an infection in an adult could be made up to contain 250 ml of sterile Ringer's solution, and about 100 mg to 10 grams of antibody. Actual methods for preparing parenterally or orally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Science, 16th ed., Mack Publishing Company, Easton, PA (1982), which is incorporated herein by reference.

The compositions containing the present homoconjugated antibodies or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease,

i.e., infection, tumor, etc., the age of the patient and the general state of the patient's immune system. Generally, the amounts will range from about 0.1 to about 50 mg of antibody per kilogram of body weight per dose, with dosages of from 5 to 25 mg of antibody per kilogram per patient being more commonly used. It must be kept in mind that the materials of the present invention may generally be employed in serious disease states, that is, life-threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and the possibility of lower "foreign substance" rejections which may be achieved by, e.g., administering allogeneic homoconjugated antibodies or chimeric homoconjugated antibodies made feasible by this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these antibodies.

In prophylactic applications, compositions containing the present antibodies or cocktails thereof are administered to a patient not already in a disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend on the patient's state of health and general level of immunity, but generally range from 0.1 to 25 mg per kilogram, especially 0.5 to 2.5 mg per kilogram. A preferred prophylactic use is for treatment of fetuses and neonates at risk from infection through their mothers. When treatment is dependent on passage through the placenta, the dosage may require adjustment to reflect the percentage of antibody which is able to pass from the blood of the pregnant female to that of the fetus.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a

quantity of homoconjugated antibody sufficient to treat the patient.

The homoconjugated antibodies of the invention may also find several uses in vitro. By way of example, the homoconjugated IgG antibodies of Example I below can be used for detecting the presence of group B streptococci or E. coli K1, for vaccine preparation, or the like.

For in vitro diagnostic purposes, the antibodies may be either labeled or unlabeled. Unlabeled homoconjugated antibodies may find particular use in agglutination assays, or they may be used in combination with other labeled antibodies (second antibodies) that are reactive with the homoconjugated antibodies, such as antibodies specific for the Fc regions. Alternatively, the antibody may be directly labeled. A wide variety of labels may be employed, such as radionuclides, particles (e.g. gold, ferritin, magnetic particles, red blood cells), fluors, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens), etc. Numerous types of immunoassays are available and are known to those skilled in the art, such as competitive and sandwich assays as described in, e.g., U.S. Pat. 4,376,110, incorporated by reference herein, and Harlow and Lane, supra.

Kits can also be supplied for use with the subject antibodies in the protection against or detection of the presence of a selected antigen. Thus, the subject antibody compositions of the present invention may be provided, usually in lyophilized form in a container, either alone or in conjunction with additional antibodies. The antibodies, which may be conjugated to a label or toxin, or unconjugated, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than

about 5% wt. based on the amount of active antibody, and usually present in total amount of at least about .0001% wt., based on the antibody concentration. Frequently it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% of the total composition. Where a second antibody capable of binding to the homoconjugated antibodies is employed in an assay, this will be present in a separate vial. The second antibody is typically conjugated to a label and formulated in an analogous manner with the antibody formulations described above.

The following examples are offered by way of illustration, not by limitation.

EXAMPLE I

Preparation of Monoclonal Antibody Homoconjugates

This example demonstrates means for preparing homoconjugates of several representative monoclonal antibodies to selected tumor and bacterial antigens. The homoconjugates were then tested in functional assays described in the examples which follow.

Homoconjugates of the following monoclonal antibodies were prepared: Monoclonal antibody D3, a human IgG_1 antibody which binds to the group B carbohydrate of group B streptococci. 5E1-G, a human IgG_1 monoclonal antibody which binds to the capsular carbohydrate of *E. coli* K1. BR64, a murine IgG_1 monoclonal antibody which binds to human carcinoma associated antigen, including colon, breast, ovary and lung carcinomas. BR64 is on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, M.D, as ATCC No. HB 9895. And BR96, also on deposit with the American Type Culture Collection as ATCC No. HB

10036, is an IgG human-murine chimeric IgG monoclonal antibody which binds to human lung and breast tumor associated antigens.

Homoconjugates of each of the antibodies were prepared using maleimidobutyrloxysuccinimide and iminothiolane according to the following protocol. Antibodies (1 mg/ml) were dialyzed overnight against a coupling buffer (0.1M Na_2HPO_4-dibasic, seven-hydrate, 0.1M NaCl, pH 7.5). One milliliter of antibody was thiolated with 2-iminothiolane-HCl (Pierce Chemical Co., 50 µl (0.5 mg) of 2-iminothiolane solution (10 mg/ml in coupling buffer) added while mixing. A second aliquot of the antibody (1 ml) was treated with N-γ-maleimidobutyryloxy-succinimide (GMBS)(Calbiochem, La Jolla, CA), 5 µl (14 µg) of GMBS solution (1 mg in 360 µl dimethylformamide (DMF). Each treated aliquot of antibody was incubated 1 hr. at room temp. and then the antibodies were run over PD-10 columns (Pharmacia) pre-equilibrated in coupling buffer. After a void volume of 2.6 ml total, antibodies were collected in double the original volume. The thiolated and GMBS-treated aliquots of antibodies were then mixed and incubated at room temp. for 5 hrs. The reactions were quenched by adding 1 µl of 25mM β-mercaptoethanol (1 µl in 560 µl coupling buffer) and incubating for 15 min. at room temp. The β-ME was stopped by adding 11 µl (11 µg) N-ethylmaleimide (Sigma Chemical Co., St. Louis) made up to 1 mg/ml in DMF. The homoconjugate preparations were dialyzed overnight in phosphate buffered saline (PBS) and separated by size-exclusion chromatography using Superose-6 and Superose-12 FPLC columns (Pharmacia, Uppsala, Sweden). The chromatograms of the FPLC columns for monoclonal antibodies D3, 5E1-G, and BR64 are shown in Fig. 1.

EXAMPLE II

Binding Activity of Homoconjugates

The ability of the tetravalent and hexavalent monoclonal antibody homoconjugates to bind antigen was compared to the binding activity of the bivalent IgG monomer antibodies. The binding of the anti-GBS homoconjugates was measured against a GBS strain (I334) bound to microtiter wells using poly-L-lysine (PLL). Equivalent protein concentrations of untreated antibody D3 monomer were compared to FPLC fractionated IgG dimer and trimer homoconjugates. Binding was assayed with biotin labeled anti-human gamma-chain specific antibodies. The results are shown in Fig. 2, where the relative binding activities of the dimer or trimer homoconjugate preparations were significantly greater than the initial IgG monomer.

To measure the binding of the anti-*E. coli* K1 antibody homoconjugates, *E. coli* strain H16 was bound to microtiter wells using poly-L-lysine. Untreated antibody 5E1-G was compared to homoconjugates of IgG dimer and trimer, prepared as described above. Equivalent protein concentrations of antibodies were reacted with the *E. coli*. Binding was assayed with biotin labeled anti-human gamma-chain specific antibodies. The results are shown in Fig. 3, where the relative binding activities of the dimer and trimer homoconjugate preparations were significantly greater than the initial IgG monomer.

To measure the binding of the anti-breast tumor antibody, BR64, and homoconjugates thereof, a breast tumor cell line, 3396, was grown adherently to microtiter wells. Untreated antibody BR64 was compared to homoconjugates of IgG dimer and trimer. Equivalent protein concentrations of antibodies were reacted with the breast tumor cells. Binding was assayed with biotin labeled anti-murine gamma-chain specific antibodies. The

results are shown in Fig. 4, where the relative binding activities of the dimer and trimer homoconjugate preparations were significantly greater than the initial IgG monomer.

To measure the binding of the anti-breast and lung tumor antibody, human-mouse chimeric BR96, and homoconjugates thereof, two breast cell lines (H3396 and H3760B) and two lung cell lines (H2987 and H2707) were used as targets. Freshly trypsinized cells were attached to microtiter plates using PLL and the ELISAs performed as follows. PLL, made up at 1 μg/ml in PBS, was adsorbed to Immulon 96-well microtiter plates by incubating 75 μl/well of the PLL solution for 1 hour at room temp. Carcinoma cell lines (cultured in IMDM with 15% FCS) were trypsinized, washed twice, and resuspended in PBS at 2 x 10^5 cells/ml. The PLL treated ELISA plates were washed 3 times with saline/Tween (all wash steps done with a gravity flow wash system). The cell suspension was added at 100 μl/well (about 20,000 cells/well) and incubated for 1 hr at 37°C. The plates were then washed 3 times with saline/Tween. Antibodies were diluted in specimen diluent (5% nonfat dry milk, 100 μl/L Foam A, 0.01% w/v thimerosal in PBS) then added to the ELISA plates (100 μl/well) and incubated for 1 hr at room temp. Following incubation, the plates were washed 3 times with saline/tween, and peroxidase-conjugated goat anti-human or mouse IgG (Tago) diluted in specimen diluent was used as a second step reagent, (100 μl/well) and incubated for 1 hr at room temp. The plates were then washed 5 times with saline/Tween, and tetramethylbenzidine (TMB) chromogen (TMB), diluted 1:100 in buffered substrate, was added (100 μl/well), and plates incubated for 20 minutes. The reactions were stopped with 100 μl/well of 3N H_2SO_4 and the plates read at dual wavelength, 450/630nm.

Untreated monoclonal antibody BR96 was compared to homoconjugated BR96 IgG dimers using approximately equivalent protein concentrations of antibody. The

results, shown in Fig. 5A-D for each of the tested tumor cell lines, indicate that the relative binding activity against the four tumor cell lines by the predominantly dimer homoconjugate preparation was greater than by the initial IgG BR96 monomer.

EXAMPLE III

Increased In Vitro Activity of Homoconjugates

As an indication of *in vivo* effectiveness, the monoclonal antibody homoconjugates to GBS were tested in an *in vitro* opsonophagocytic assay. Homoconjugates to *E. coli* K1 were tested for functional activity in two types of opsonization assays described below. Homoconjugates of BR64 were tested for *in vitro* function in a complement dependent cytotoxicity assay, and homoconjugates of BR96 were tested in a complement independent cytotoxicity assay.

Opsonization of GBS by Homoconjugates of D3

The opsonophagocytic assays for GBS were performed as follows. Bacteria were prepared by inoculating 10 ml of tryptic soy broth (TSB) with 50 µl of an overnight broth culture. The tubes were incubated at 37°C on a shaker for 3 hours at which time 1.5 ml of the culture was centrifuged for 1 min. at 10,000 x g, the spent culture media discarded, and the pellet was suspended in 3.5 ml of Hank's balanced salt solution containing 0.1% gelatin and 5 mM HEPES (HBSS/Gel). The bacterial concentrations were adjusted to about 3×10^4 bacteria/ml by measuring the $O.D._{600}$ and making the appropriate dilutions (approximately 1:50,000). Human neutrophils were isolated according to van Furth and Van Zwet ("*In Vitro* Determination of Phagocytosis and

Intracellular Killing by Polymorphonuclear and Mononuclear Phagocytes," in Handbook of Experimental Immunology, Vol. 2, D.M. Weir, ed., 2nd edition, Blackwell Scientific Publications, Oxford, 36.1-36.24 (1973)) with several modifications. Buffy coat from 5 ml of heparinized blood diluted 1:2 with PBS was underlayed with Lymphocyte Separation Medium and centrifuged. The red blood cell (RBC) pellet was washed once with RPMI 1640 medium and resuspended in an equal volume of 37°C PBS. Twenty-five ml of this suspension was added to 25 ml of 2% dextran (in 37°C PBS) and the contents gently but thoroughly mixed end over end. After a 20 min. incubation at 37°C to allow the RBC's to sediment, the supernatant (containing neutrophils) was removed, washed twice in 4°C PBS, once in HBSS/Gel, and suspended in same to $5x10^7$ neutrophils/ml. For the complement source used with GBS, human serum was thrice adsorbed with live bacteria (Bjornson, A.B. and Michael, J.G., J. Inf. Dis., 130 Suppl:S119-S126 (1974)) corresponding to the organisms used in the assay.

For the assay, into 1.5 ml sterile polypropylene microfuge tubes were added 250 µl antibody (test homoconjugates or monomer) preparation in 10% fetal calf serum in HBSS/gel with HEPES and 100 µl bacterial suspension (about 3×10^4 bacteria/ml). After 30 minutes at 37°C, 150 µl containing 75 µl complement, 50 µl neutrophils (5×10^7 ml), and 25 µl HBSS/gel were added. The mixtures were incubated on a rotator for 60 minutes at 37°C, after which they were placed into an ice water slurry. After 10 minutes, 20 µl from each tube was added to a 100 mm petri dish containing 3 ml of solidified 0.5% tryptic soy broth agarose, followed by incubation at 37°C. After 18 hours the colonies were enumerated and the data was reported as colony forming units (CFU) for each condition.

The results for homoconjugates of D3 are shown in Fig. 6, where the dimer and trimer required much less

antibody, on a nanogram protein basis, to opsonize the GBS strain tested when compared to the initial IgG monomer.

As a further indication of in vivo effectiveness, homoconjugates prepared with an additional monoclonal antibody to the group B carbohydrate of GBS (D3, produced as generally described in Raff et al., J. Infect. Dis. 163:346 (1991) and PCT patent publication WO 91/06305, each of which is incorporated herein by reference) were tested in in vitro opsonophagocytic assays against two GBS strains, M94 and I334. The results of the assays are shown in Fig. 7, where it is evident that the anti-GBS D3 homoconjugates resulted in increased opsonization of the GBS human clinical isolates. Again, these results suggest that the homoconjugates will significantly increase the in vivo protective activities of the antibodies when compared to the parental IgG monomeric monoclonal antibodies.

Opsonization of E. coli K1 by 5E1-G Homoconjugates

To isolate human neutrophils, heparinized human blood (5 ml) was layered onto 3.0 ml of Mono-Poly Resolving Medium (MPRM, Flow Labs) in polystyrene tubes and centrifuged for 30 minutes at 300 x g at room temp. After centrifugation, three cell layers were evident, with the middle layer containing neutrophils. The serum and top cell layer were removed and discarded, the neutrophils collected and added to a 50 ml tube containing pre-warmed PBS. The neutrophils were centrifuged for 10 minutes at 300 x g at room temp., the supernatant discarded and the cell pellet resuspended with 10 ml tissue culture media (RPMI-1640) containing 0.5% gelatin, and the cell concentration adjusted to 5 x 10^6 cells/ml.

The assays were performed as follows. To luminometer tubes (LKB Nuclear) were added 100 μl

containing appropriate test (5E1-G) or control IgG monoclonal antibody monomer to P. aeruginosa flagella, 100 µl log phase growth bacterial suspension (OD_{660} =0.02), and 100 µl diluted bacteria-adsorbed human serum complement, final concentration 3.3%. The complement was thawed just prior to use and received 5 µl of 2 M $CaCl_2$/ml. The tubes were placed into a prewarmed LKB Luminometer which allows 24 tubes to be run on a continuous reading cycle. After 30 minutes in which the tubes were warmed and periodically mixed, 100 µl of neutrophils (5×10^6/ml) and 600 µl of 10^{-4} M Luminol in Hank's Balanced Salt Solution were added. Counting sessions for 25 continuous cycles, which corresponded to ~80 minutes for 24 sample tubes, were initiated. The chemiluminescence intensity was displayed as millivolts (mV) with

$$\text{signal:noise} = \frac{\text{mV values for tubes containing the test antibody}}{\text{average of tubes containing negative antibody.}}$$

The results of the assays are shown in Fig. 8, where it is evident that the homoconjugates resulted in increased opsonization of the E. coli organisms than the initial IgG monomers. The homodimer and homotrimer of 5E1-G were significantly more opsonic than the 5E1-G IgG monomeric form. As the opsonophagocytic assays are typically predictive of in vivo ability to protect animals (see, e.g., U.S. Pat. No. 4,970,070, incorporated herein by reference), these results suggest that the dimer and trimer homoconjugates will significantly increase the in vivo protective activities of the antibodies when compared to the parental IgG monomeric antibody.

As a further confirmation of in vitro efficacy, and thus in vivo activity, the monoclonal antibody homoconjugates to E. coli K1 were tested in in vitro opsonophagocytic assays, as described above, against two

additional E. coli K1 strains, H16 and A14. As shown in Fig. 9, the anti-E. coli K1 homoconjugates resulted in increased opsonization of the human clinical isolates, suggesting that the predominantly dimer homoconjugate preparations will significantly increase the in vivo protective activities of anti-E. coli K1 monoclonal antibodies.

Complement Dependent Cytotoxicity BR64 Homoconjugates

In vitro functional assays were also used to demonstrate the increased functional activity of the anti-tumor antigen homoconjugated monoclonal antibodies. For testing BR64 homoconjugates, target tumor cells (H3630) were labeled with ^{51}Cr by incubation 1 x 10^6 cells/0.3 ml tissue culture media in 100 μCi of ^{51}Cr for 1 hour at 37°C, 6% CO_2. After washing to remove excess ^{51}Cr, 2 x 10^4 labeled cells in 67 μl media (RPMI-1640 plus 15% fetal bovine serum) were added per microtiter plate well. Next, 67 μl of the appropriately diluted test monomer (BR64), a negative control monomer (Mab 96.5), or homoconjugated (dimer) monoclonal antibody was added to duplicate wells. Finally, 67 μl of freshly thawed human serum complement was added to each well, the plates covered with parafilm and incubated at 37°C for 4 hours. After incubation, plates were centrifuged at 400 g for 10 minutes, and 100 μl of supernatant was removed from each well and placed in 12 x 75 mm polystyrene tubes. The tubes were counted in a gamma counter. The following controls were included in each assay:

Table I

Well	Media	Serum	Diluted Antibody	Target Cell
Spontaneous Release	134[§]	-	-	67
Complement Toxicity	67	67	-	67
Total Incorporation	134	-	-	67
Maximum Release*	67	-	-	67
Antibody Alone	67	-	67	67

[§]Amounts are expressed as µl/well.

*Prior to incubation of the assay, these are the only wells which contain less than 201 µl, because the wells later receive 67 µl Triton X-100 to lyse labelled target cells.

The percentage kill (% kill) was calculated from the following formula:

$$\frac{\text{[Test (mean CPM)- Hc' control (mean CPM)]}}{\text{Total incorporation [mean CPM - Hc' control (mean CPM)]}} \times 100 = \% \text{ kill}$$

where CPM is counts per minute as average of duplicate samples obtained from measurement in gamma counter and Hc' is Complement Toxicity control.

The results of the assays are shown in Fig. 10, where it is evident that the homoconjugated BR64 resulted in eight times greater killing of the targeted tumor cells than the initial IgG BR64 monomer. The CDC assay is generally predictive of <u>in vivo</u> ability to protect animals against tumors. These results suggest that the homoconjugates will significantly increase the utility of such antibodies <u>in vivo</u> against tumors, particularly when compared to parental IgG monomer antibodies.

Increased Complement Independent Cytotoxicity of Chimeric BR96 Homoconjugates

Target tumor cells (H3396) at 5 x 10^5 cells/tube were mixed with 100 µl of test antibody and were incubated at 37°C for 30 minutes. Cells were pelleted and mixed with the appropriate concentration of propidium iodide (Sigma, 10 µg/tube). Propidium iodide is a DNA reactive stain that only penetrates the membrane of dead or dying cells. Therefore, by quantitating the number of fluorescent cells within the population, the number of dead cells can be determined (Hellstrom et al., Cancer Res., 50:2183-2190 (1990)). After incubation for 10 minutes, the cells were washed in tissue culture media containing 15% fetal calf serum, resuspended in same, and placed on ice. The cells were analyzed for fluorescence on an EPICS Fluorescence Activated Cell Sorter which quantitates live and dead cells on the basis of fluorescence and size (small and large represent dead and live cells, respectively). The results (Fig. 11) showed that the BR96 homoconjugate dimers were dramatically more effective in killing the tumor cells than the initial monomer. These results suggest that the homoconjugates will significantly increase the utility of such antibodies in vivo against tumors.

EXAMPLE IV

In Vivo Protection Against E. coli K1 Infection in Neonatal Rats Using IgG Homoconjugates

Outbred Sprague-Dawley rat pups less than 48 hours old (housed with their mothers) were injected intraperitoneally with approximately 72 E. coli K1 organisms, and 2 hours later received 1 or 5 μg of dimer homoconjugates of 5E1-G, or 100 μg of monomeric 5E1-G antibody, or control IgG and IgM antibodies. In all experiments, the rat pups were examined daily for symptoms and were scored for survival. The results of the experiments, shown in Table II below, demonstrate that 5 μg of the dimer homoconjugates of 5E1-G antibody protected significantly more animals from death when compared to animals receiving twenty times the amount (100 μg) of monomeric antibody.

Table II

Protection by Homoconjugates Against E. Coli K1 Infections

Antibody (per rat)	Dose (per rat)	n (rats/group)	%Survival (Survivors/Challenged)	p value
5E1-IgM	20 ng	26	100%	<0.001*
5E1-IgG Monomer	100 μg	15	40%	<0.01
5E1-IgG Conjugate	5 μg	14	78%	<0.01
5E1-IgG Conjugate	1 μg	14	29%	<0.05
21B8 (Negative control)	100 μg	24	0	
No antibody control	-	25	0	

*Based on survival in experimental group versus survival in negative control and controls receiving no antibody.

EXAMPLE V

Transplacental Passage of Homoconjugated Antibody to Fetuses of Pregnant Rats

The ability of the homoconjugated IgG antibody to pass through the placenta and into the fetus, and thus into the subsequently delivered offspring, was compared with the monomeric antibody. An infant rat model was used as an animal model. Similar rat models have been used to predict the transplacental passage of antibody and other molecules to human fetuses. See generally, Brambell, Frontiers Biol. 18:234-276 (1970).

Two to three days prior to their anticipated delivery date, pregnant rats were injected intravenously with 40 μg of either monomeric 5E1-G IgG (monomer) (Dams 1 and 2) or homoconjugated dimeric IgG (Dams 3 and 4). Blood samples were collected from the dams two hours after antibody administration and on the day of delivery, and from the neonatal rats just after birth. Total human IgG and human IgG anti-E. coli K1 antibody were determined in each blood sample using individually designed quantitative binding assays (ELISA's). By using anti-human IgG-specific enzyme-labeled secondary antibodies, rat IgG was neither detected nor interfered with the quantitation of the injected human IgG.

The amount of transplacentally passaged antibody was determined as follows. Anti-human gamma chain antibody was attached to microtiter plates using carbonate buffer. After adding diluted serum samples from the dams or pups, binding was assayed with biotin labeled anti-human gamma chain-specific antibodies. Since one group of dams received only conjugated antibody, any human IgG detected in pup sera should be transplacentally passed homoconjugate.

The experiments showed that the monomeric and homoconjugated IgG antibodies were transplacentally

passed with approximately equal efficiency. Therefore, the homoconjugated IgG monoclonal antibody should be useful when administered prophylactically to pregnant females at risk of having a neonate with an increased likelihood of developing a life-threatening infection, such as by E. coli K1 in the case of the present embodiment. The data also support the use of these homoconjugates in transplacental treatment of a variety of other infections and tumors.

EXAMPLE VI

Transplacental Passage of Homoconjugated Monoclonal Antibody to Group B Streptococci

This Example demonstrates the transplacental passage of homoconjugated monoclonal antibody D3.

The experiments were performed as generally described in Example V for the homoconjugated monoclonal antibody to E. coli K1.

The results, shown in Table III, below, indicated that both the monomeric and homoconjugated IgG antibodies were transplacentally passed.

TABLE III: Transplacental Passage of Homoconjugated Antibody From Pregnant Rats to Their Neonates

Source	Time Post Injection	Antibody Injected	
		Homoconjugate	Monomer
Dams	2 Hours	1.45±0.4[a]	2.4±0.3
Dams	3 Days (Day of Delivery)	0.14±0.04	0.11±0.03
Pups	Day of Delivery	0.41±0.1	0.70±0.2

[a] Concentration of human IgG in rat serum (μg/ml)

Accordingly, the homoconjugated IgG monoclonal antibody is useful administered prophylactically or therapeutically to pregnant females likely to deliver a neonate susceptible to developing or already having an infection, such as by group B streptococci or E. coli K1. The present invention also makes possible the use of the homoconjugates in transplacental treatment of a variety of other infections and tumors.

EXAMPLE VII

In Vivo Protection Against Group B Streptococcal Infection With IgG Homoconjugates

This Example describes the use of homoconjugates of the D3 monoclonal antibody to protect against group B streptococcal infection in vivo, consistent with and confirming the results of the in vitro opsonophagocytic assays.

As generally described for the E. coli K1 protection studies described in Example IV above, outbred Sprague-Dawley rat pups less than 48 hours old (housed with their mothers) were injected intraperitoneally with approximately 100 GBS organisms two hours after receiving an intraperitoneal injection of either 20, 4, 0.8 or 0.2 μg of predominantly dimer homoconjugate preparations, 80, 20, or 4 μg of monomeric D3, or control IgG. In the experiments, rat pups were examined daily for seven days and were scored for symptoms and survival. The results from two experiments (data pooled, 25 animals/group), shown in Fig. 12, demonstrate the increased in vivo protective activity against GBS of the dimer homoconjugates of human monoclonal antibody D3 compared

to the initial IgG monomer. As little as 4 μg of homoconjugated dimer protected animals nearly as well as that conferred by 80 μg of monomer.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition which comprises covalently cross-linked homoconjugated monoclonal antibodies having at least two IgG antibody molecules which bind to the same antigenic determinant, and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, the homoconjugated monoclonal antibodies having two antibody molecules.

3. The pharmaceutical composition of claim 1, the homoconjugated monoclonal antibodies having three antibody molecules.

4. The pharmaceutical composition of claim 1, wherein the antibodies are cross-linked by disulfide bonds.

5. The pharmaceutical composition of claim 1, wherein the antibodies are human.

6. The pharmaceutical composition of claim 1, wherein the antibody molecules are murine.

7. The pharmaceutical composition of claim 1, wherein the antibody molecules are murine-human chimerics.

8. The pharmaceutical composition of claim 5, wherein the human antibody heavy chain is an IgG_1.

9. The pharmaceutical composition of claim 1, which is protective against infection due to E. coli K1.

10. The pharmaceutical composition of claim 1, which is protective against infection due to group B streptococci.

11. The pharmaceutical composition of claim 1, wherein the homoconjugated monoclonal antibodies bind to a tumor associated antigen and inhibit growth of breast tumor cells.

12. The pharmaceutical composition of claim 1, wherein the homoconjugated monoclonal antibodies are capable of crossing the placenta.

13. The pharmaceutical composition of claim 1, wherein the constant regions of the light and heavy chains of the antibody molecules are human.

14. The pharmaceutical composition of claim 1, wherein the cross-linked antibodies are derived from the same cell line.

15. A method of treating a patient having a disease related to an antigen, the method comprising administering to the patient a therapeutically effective amount of homoconjugated monoclonal antibodies including at least two covalently cross-linked IgG antibody molecules which bind to the same determinant of the antigen.

16. The method of claim 15, wherein the antigen related disease is group B streptococcal infection.

17. The method of claim 15, wherein the antigen related disease is E. coli K1 infection.

18. The method according to claim 15, wherein the homoconjugated monoclonal antibodies are administered to a pregnant patient and the homoconjugates are able to pass through the placenta into fetal circulation.

19. The method according to claim 18, wherein the homoconjugated monoclonal antibodies are able to treat the fetus for the antigen related disease.

20. The method of claim 19, wherein the antigen related disease is infection by group B streptococci or E. coli K1.

21. The method of claim 15, wherein the antigen related disease is breast tumor and the homoconjugated monoclonal antibodies bind to a breast tumor associated antigen.

22. In a method for therapeutic administration of monoclonal antibodies to a patient for treatment of a disease related to an antigen, the improvement which comprises administering to the patient homoconjugated monoclonal antibodies including at least two covalently cross-linked IgG antibody molecules which bind to the same antigenic determinant of the antigen.

Chromatograms of Homoconjugate Mixtures

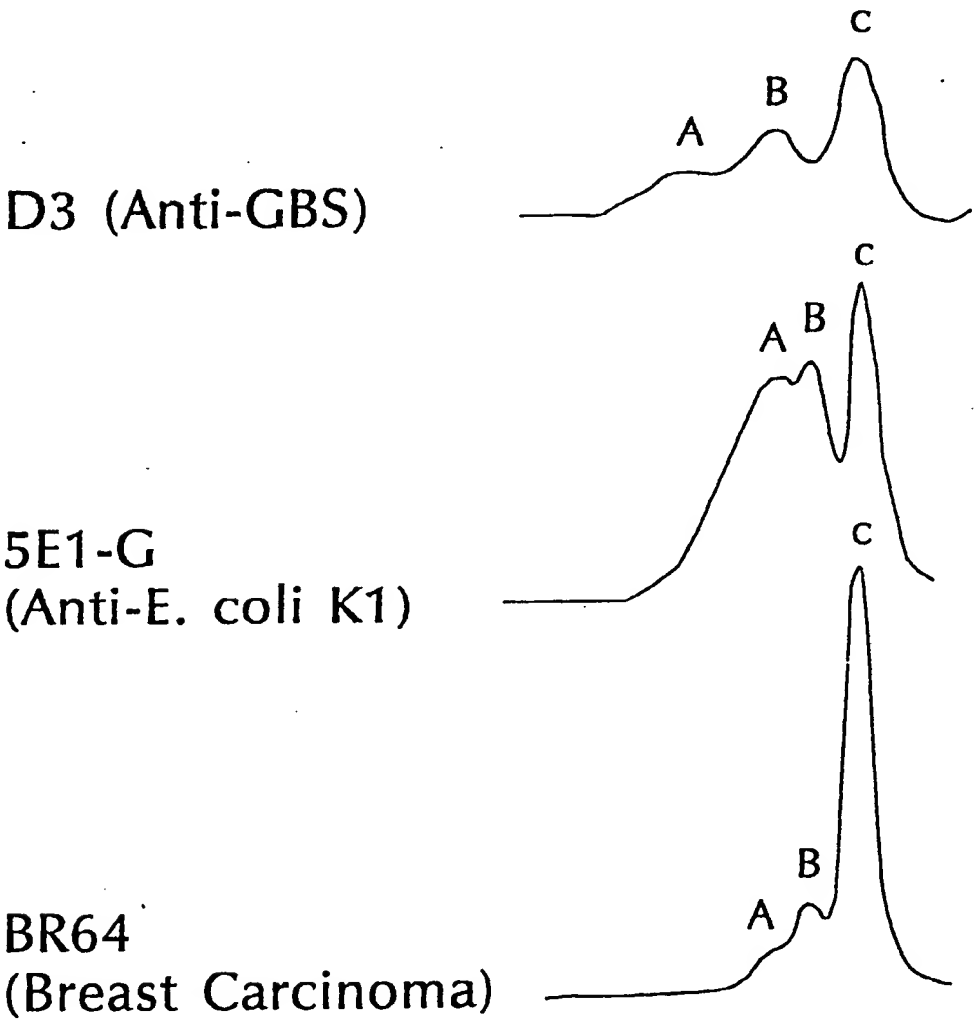

Figure 1

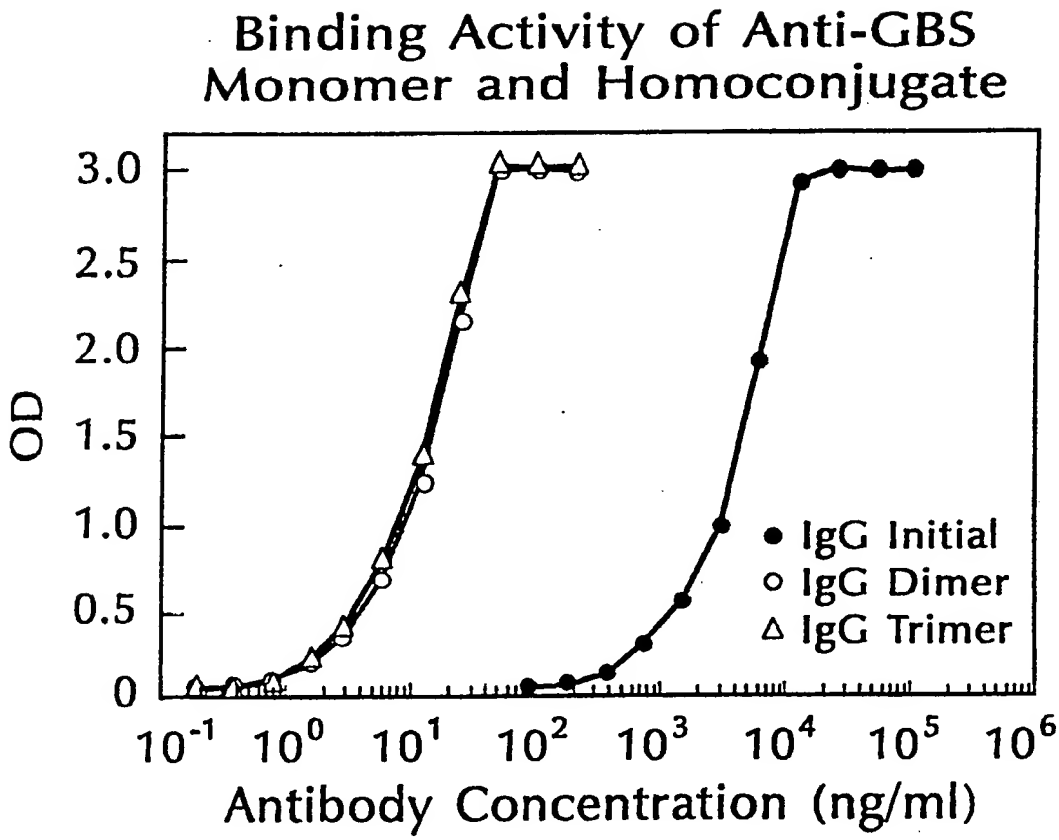

Figure 2

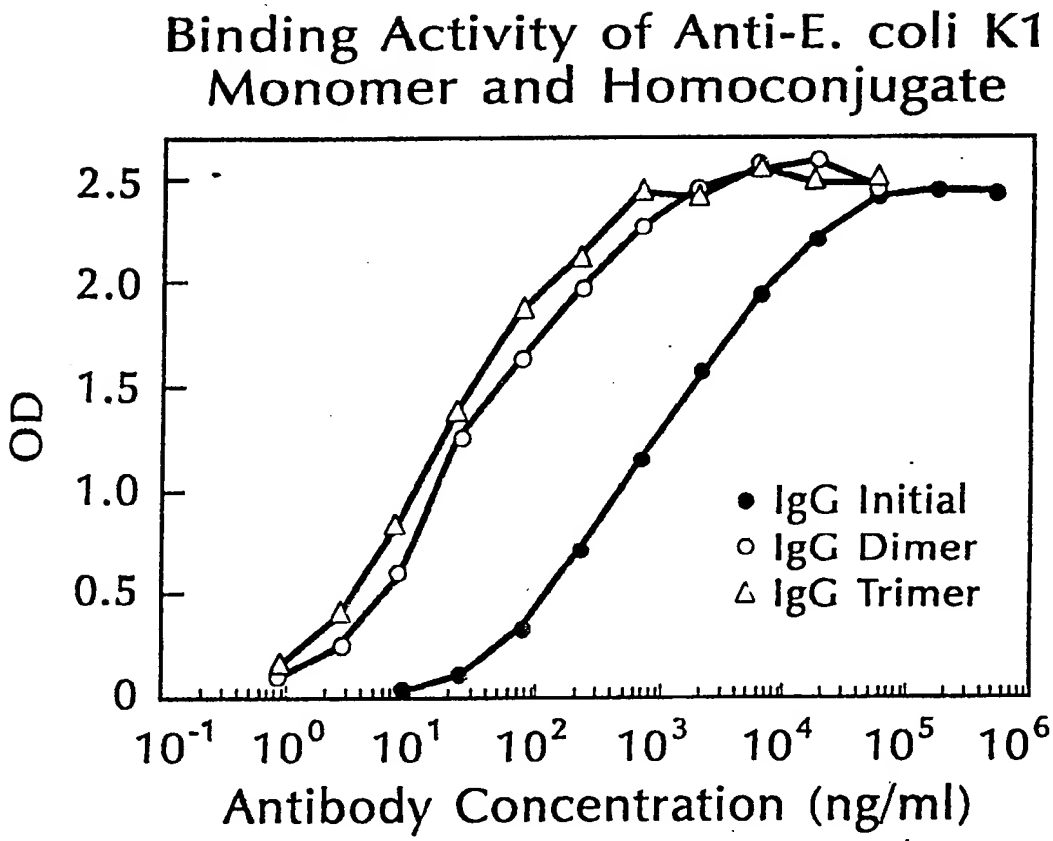

Figure 3

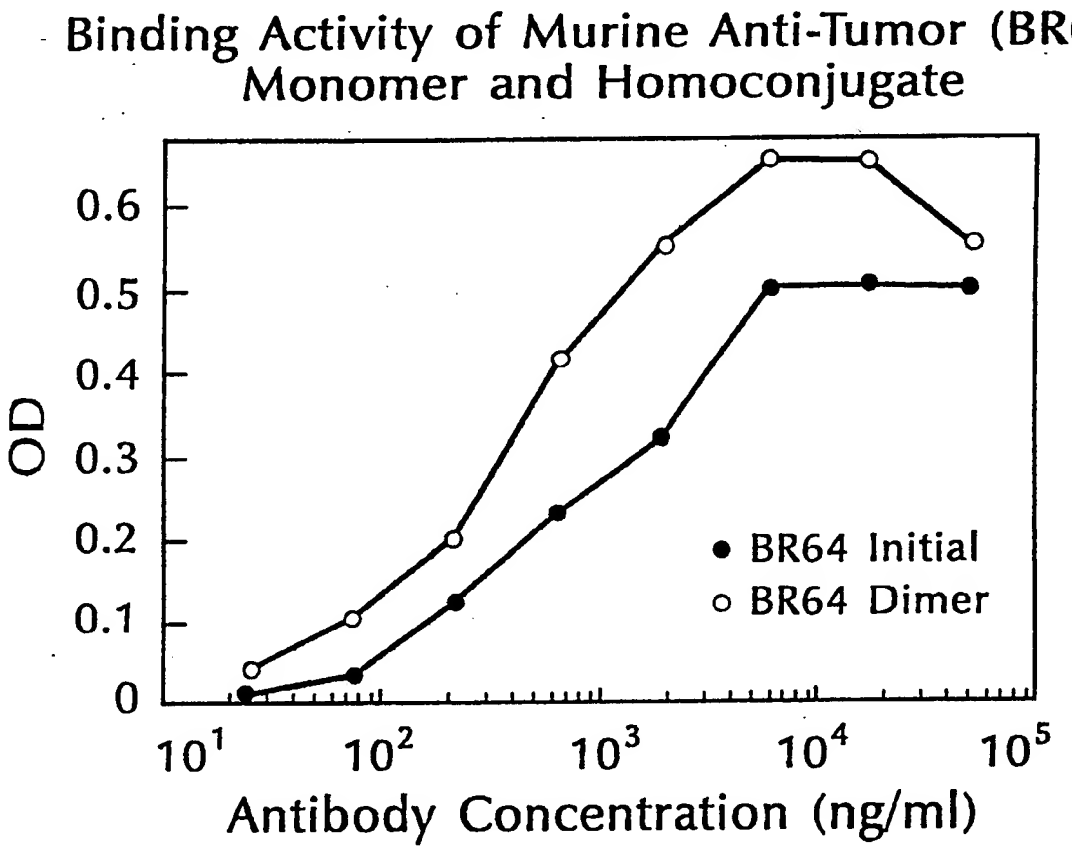

Figure 4

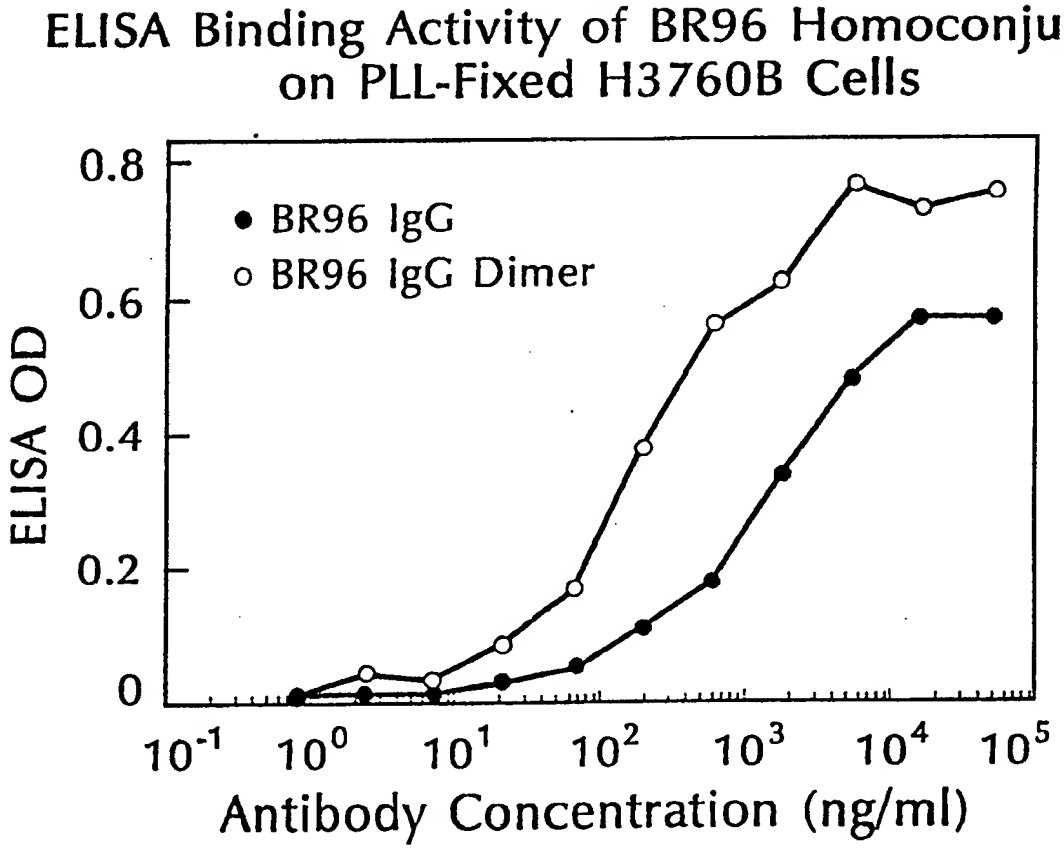

Figure 5A

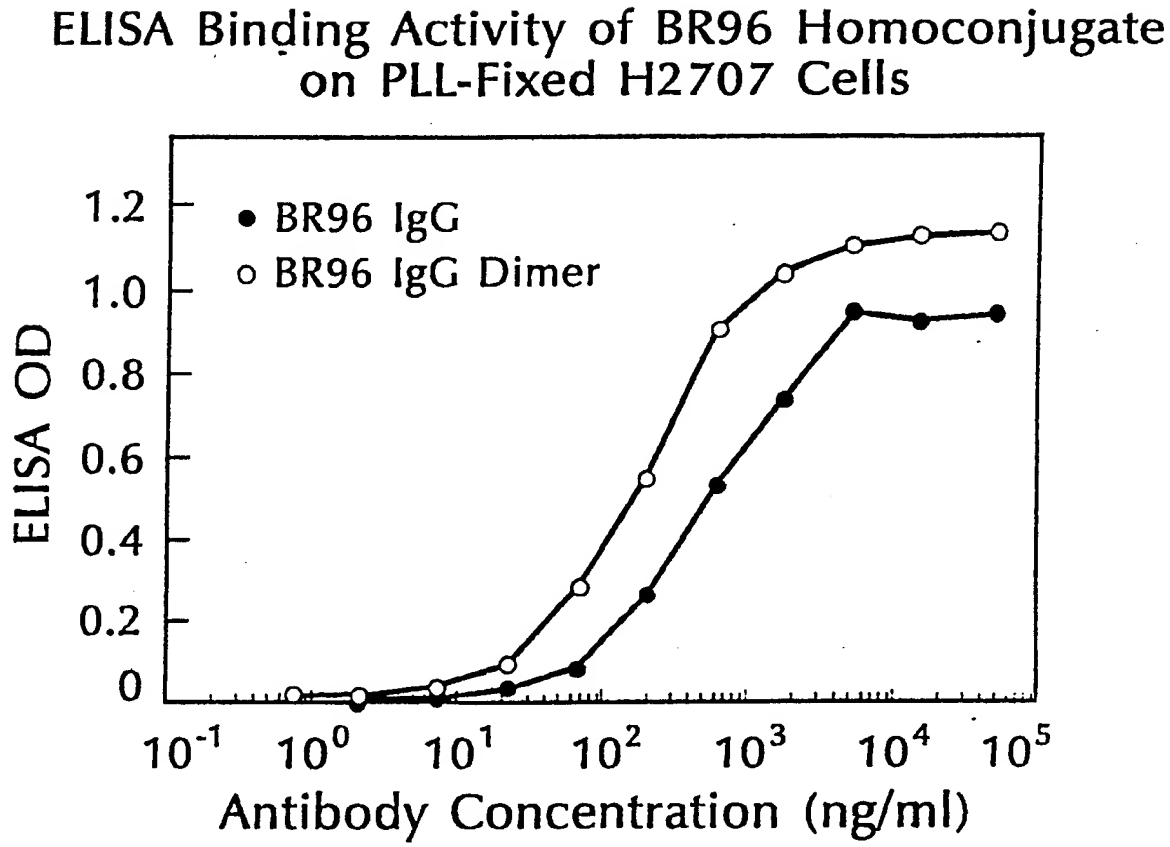

Figure 5B

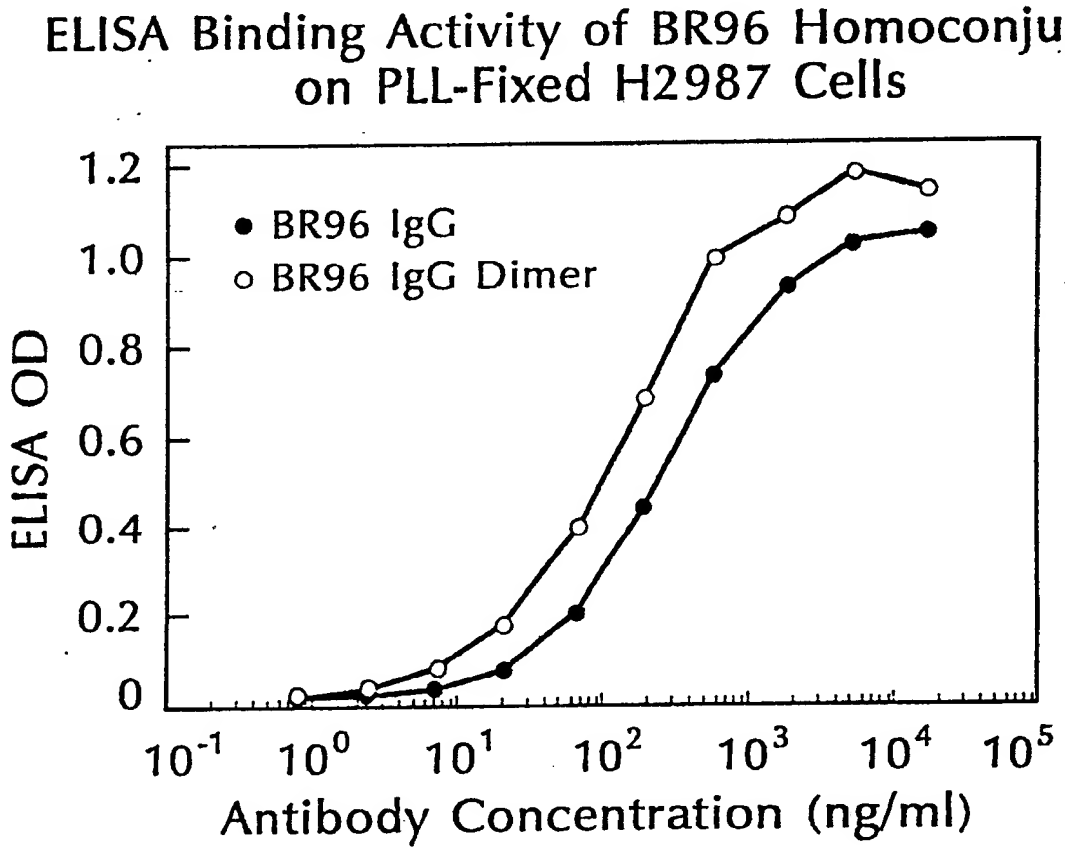

Figure 5C

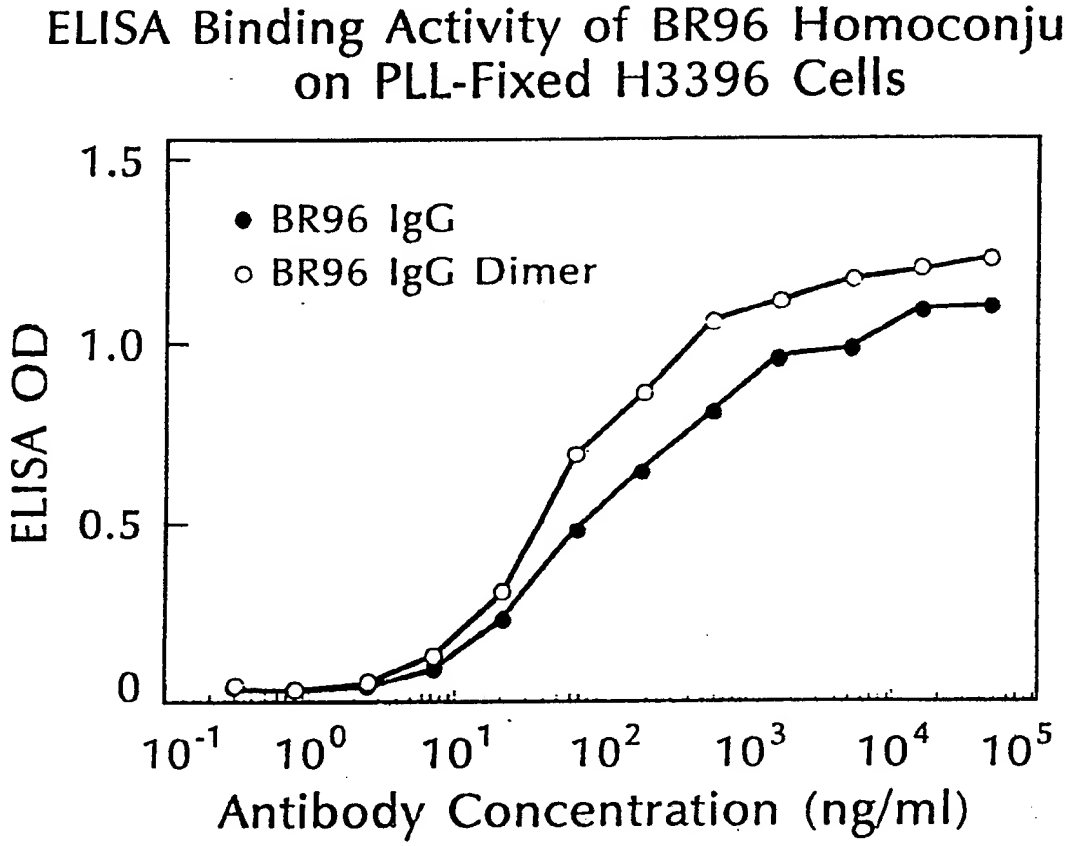

Figure 5D

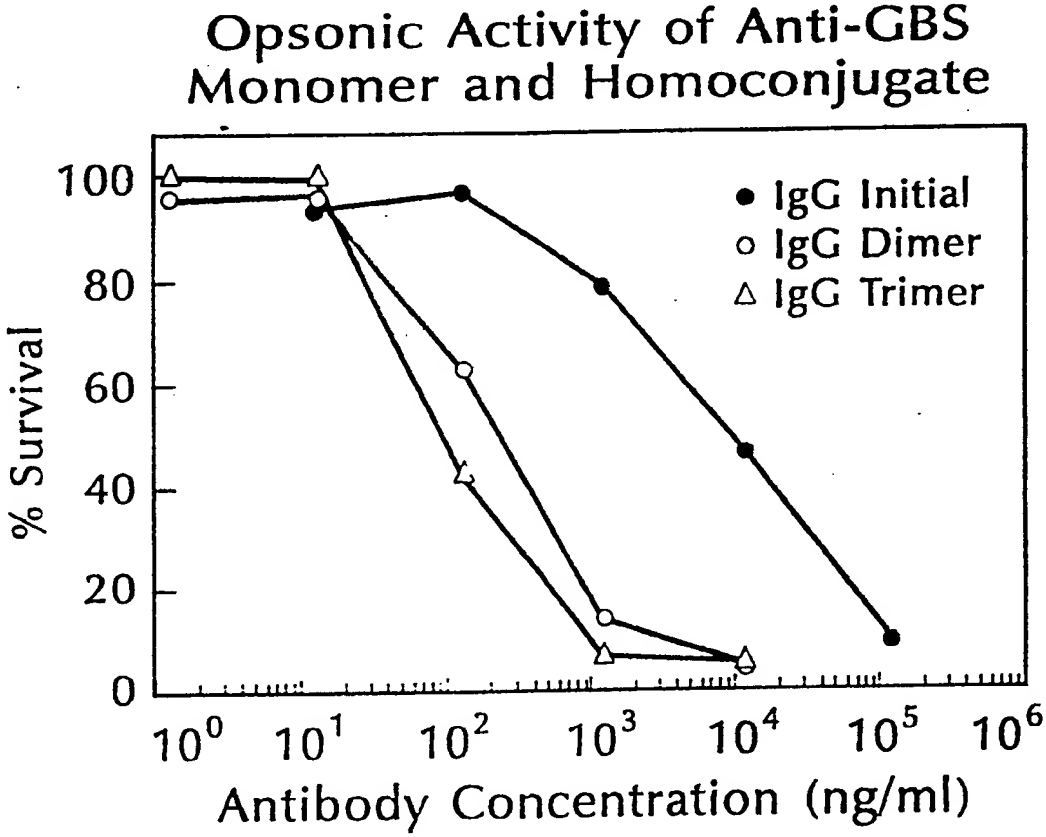

Figure 6

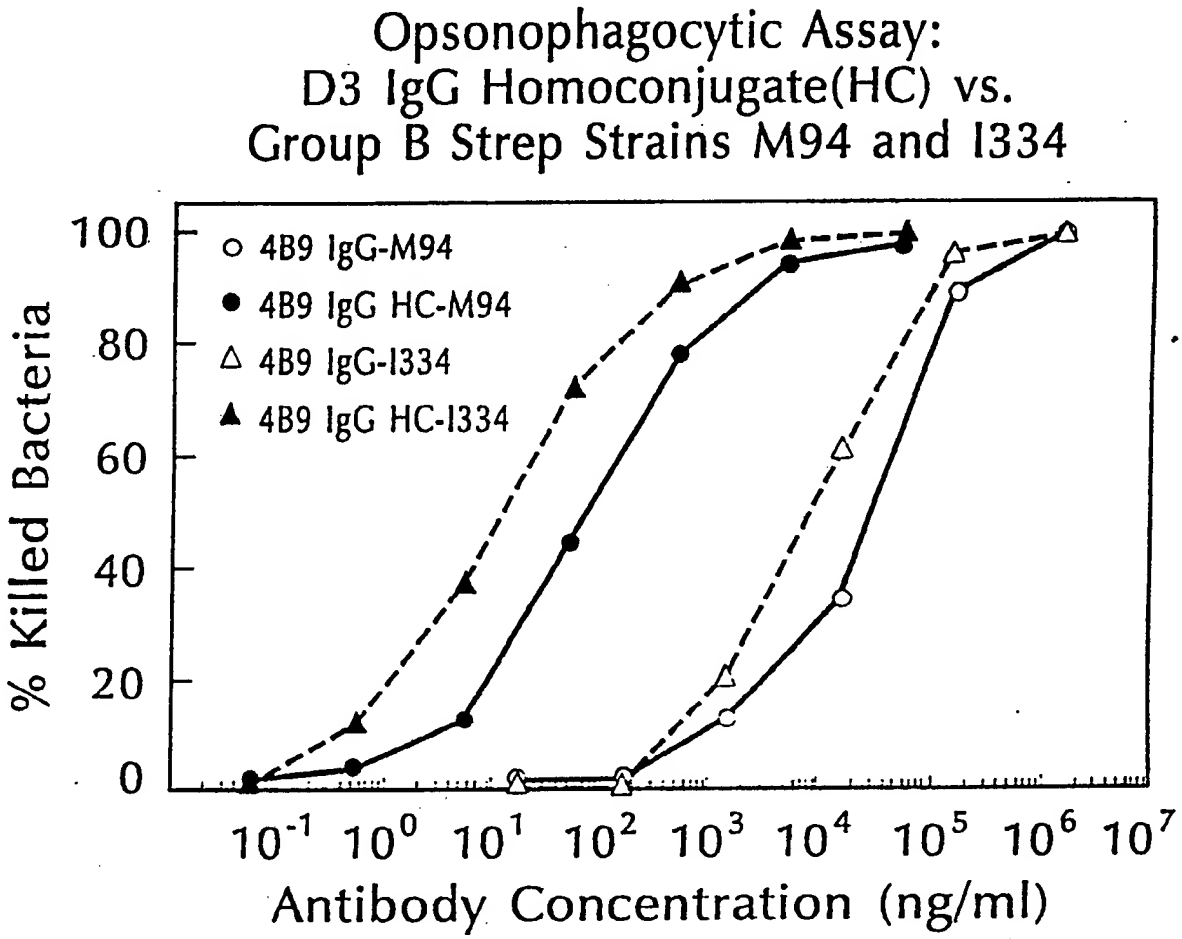

Figure 7

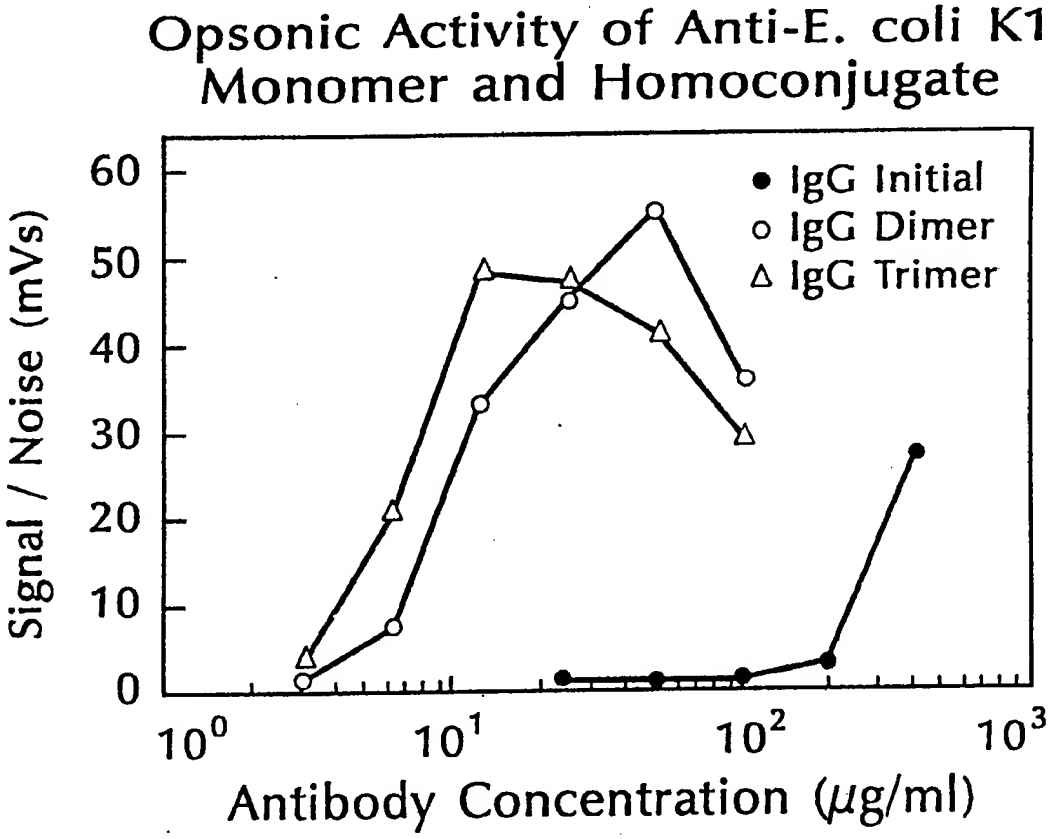

Figure 8

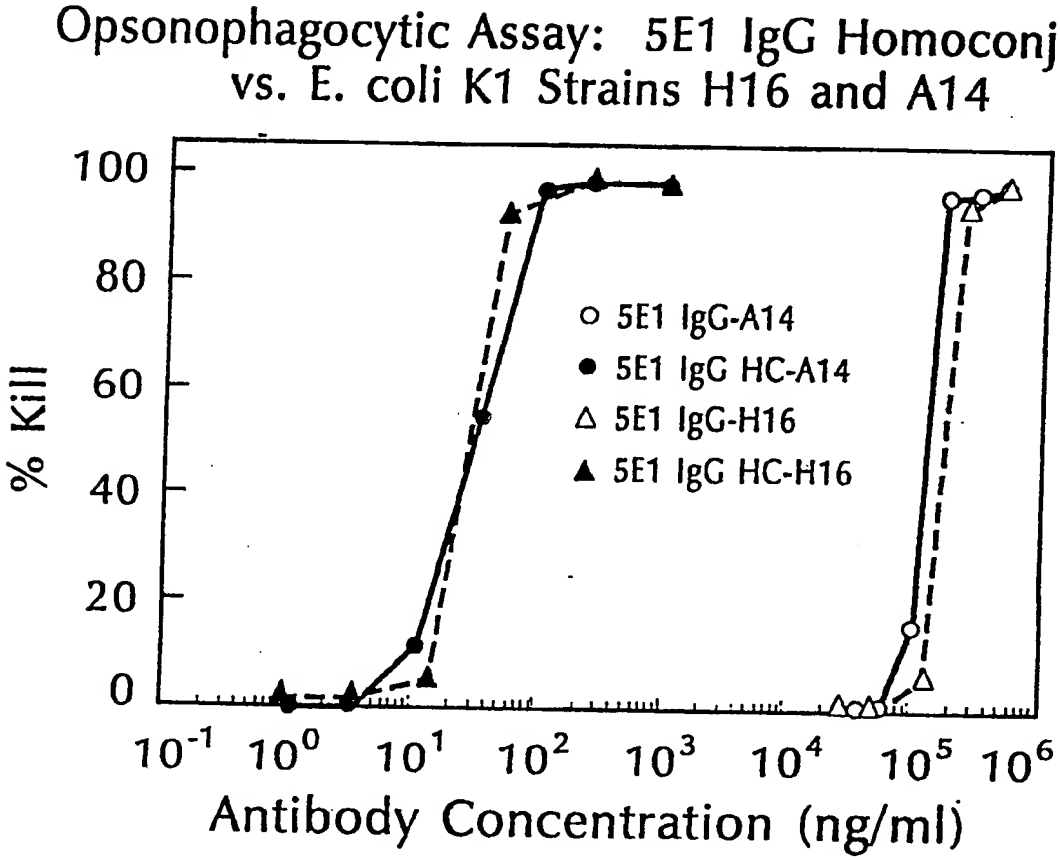

Figure 9

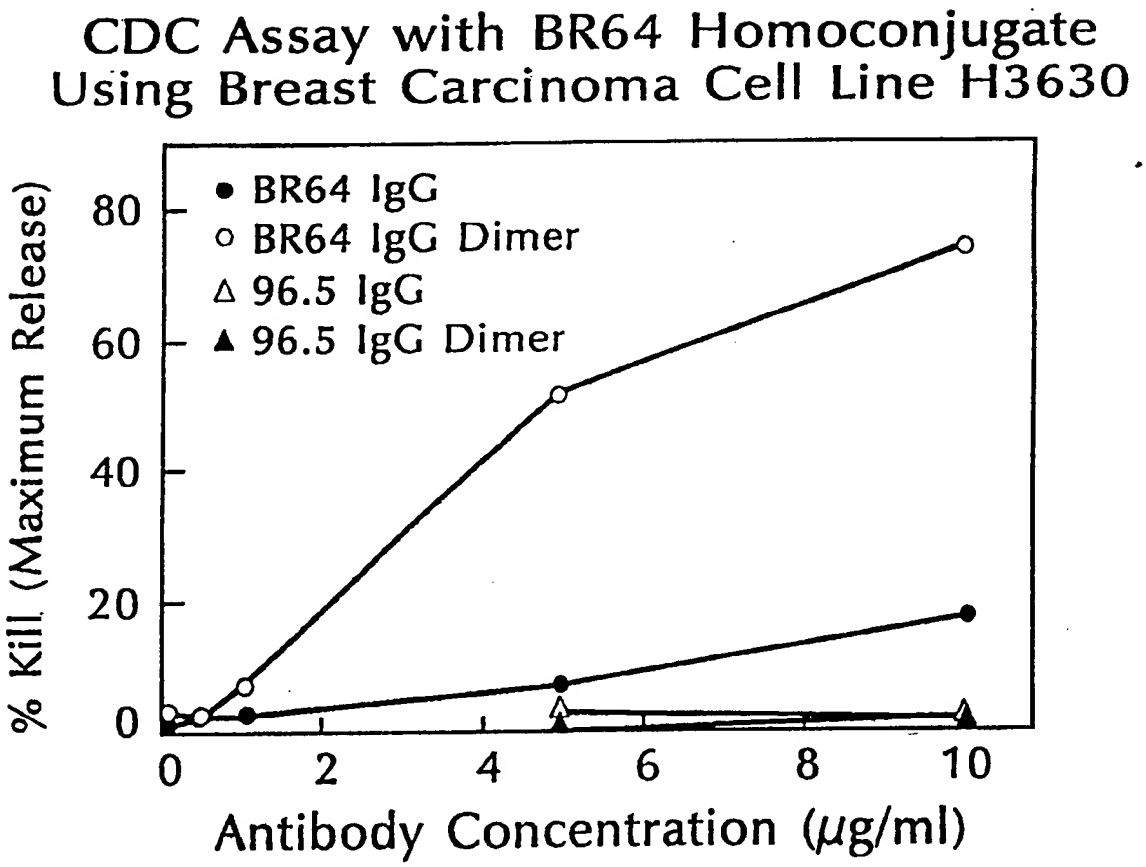

Figure 10

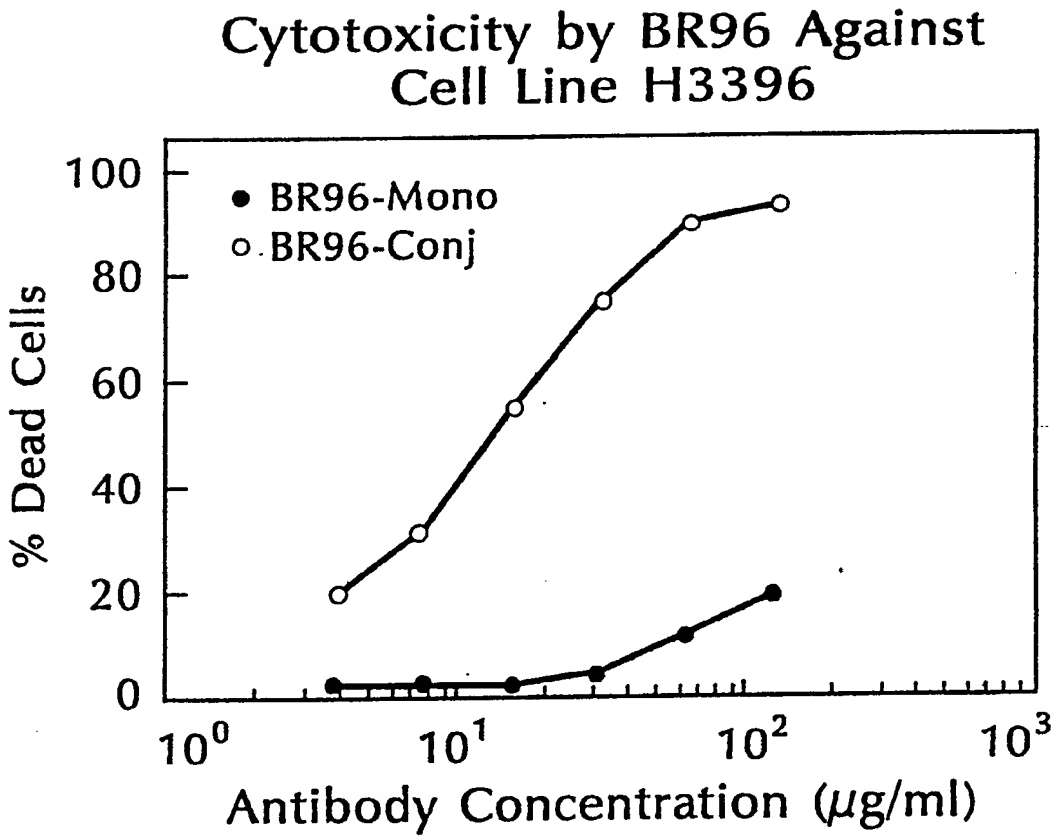

Figure 11

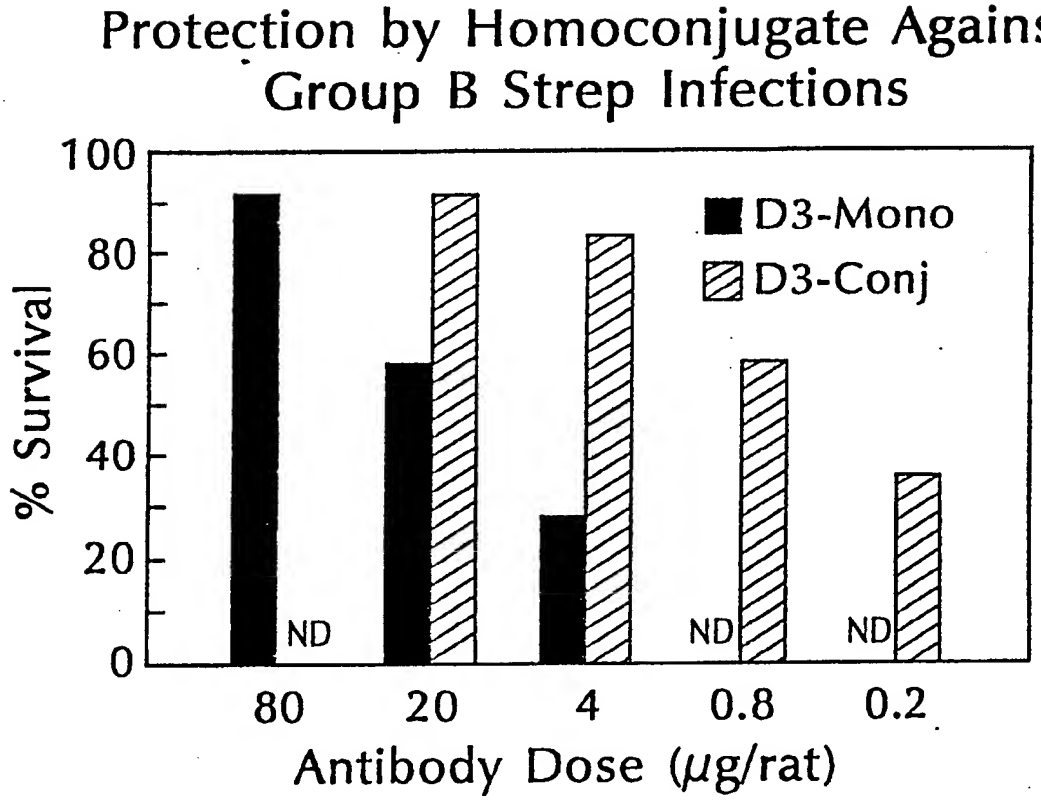

Figure 12

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06195

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/395
U.S. CL.: 424/85.8

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	424/85.8; 530/387; 435/240.27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

DATABASES: **DIALOG** (Files 5, 155, 73, 72, 76, 159, 399, 440, 144, 34)
USPTO AUTOMATED PATENT SYSTEM (File USPAT; 1971-1990)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	J. EXP. MED., Volume 168, issued September 1988, **RAFF, H.V., ET AL.**, "Human Monoclonal Antibodies To Group B Streptococcus", pages 905-917, see the entire document.	1-22
Y	J. CHROMATOGRAPHY, Volume 489, Issued 1989, **BUSH, D.A. ET AL.**, "ISOELECTRIC FOCUSING OF CROSS-LINKED MONOCLONAL ANTIBODIES", pages 303-311, see the entire document.	1-14
Y	**V. GHETIE, ET AL.**, METHODS IN ENZYMOLOGY, Volume 92, issued 1983, pages 523-543, "Preparation and Applications of Multivalent Antibodies with Dual Specificity", see the entire document.	1-14
Y,P	SCIENCE, Volume 252, issued 03 May 1991, **SHUFORD, W. ET AL.**, "Effect of Light Chain Oligomerization and in vivo Efficacy", pages 724-727, see the entire document.	1-22

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

04 October 1991

Date of Mailing of this International Search Report

04 NOV 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Susan L. Futrovsky

Susan L. Futrovsky (vsh)

Form PCT/ISA/210 (second sheet) (Rev.11-87)